The influence of human immunodeficiency virus and early antiretroviral therapy on the immunology and virology of infants and children

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Declaration

I, Helen Anne Payne, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

In children infected with human immunodeficiency virus (HIV) CD4 T-cell loss results in profound immunodeficiency and susceptibility to infection, however loss of CD4 T-cells can be partially reversed by antiretroviral therapy (ART). Although the Children with HIV Early Antiretroviral Therapy (CHER) trial demonstrated significant reduction in morbidity and mortality from early ART among South African infants, the immunological and virological mechanisms by which this survival advantage occurs are not completely understood.

The work described here used stored specimens of peripheral blood mononuclear cells and plasma from HIV-infected children in CHER and HIV-uninfected children from the Child Wellness Clinic, a cross-sectional study of healthy South African children. The overall aims were to explore the influence of HIV and ART on immunophenotypes, thymic and naïve B-cell output, HIV serostatus and HIV proviral DNA.

The main outcomes included: development of haematological and immunological reference ranges for South African children; and an observation of a naïve/memory T-cell ratio of 1:1 within the first decade of life in South African children compared with the third decade in industrialised countries. Trajectories of thymic and naïve B-cell output were created for healthy South African children. Overall early ART sustained thymic output and increased naïve B-cell output in CHER participants compared to healthy controls. Higher CD4 counts were significantly associated with increased thymic output, and deferred/interrupted ART or poor clinical outcome were

associated with lower thymic and naïve B-cell output. Early ART and continuous HIV RNA suppression reduces HIV proviral DNA, however despite adequate viral suppression, immunological disturbances persist. Finally, almost half children receiving early ART were HIV-antibody seronegative by 2 years.

These insights develop understanding of the interplay between the immunology and virology of HIV-infected children on ART, and could support further research to advance ART-strategies and optimise the immunological fitness and clinical health of HIV-infected children.

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List of Abbreviations

ABI	Applied Biosystems Informatics
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
ART-Def	CHER trial arm 1: participants randomised to deferred ART
ART-40W	CHER trial arm 2: participants receiving early limited ART for 40 weeks
ART-96W	CHER trial arm 3: participants receiving early limited ART for 96 weeks
BD	Becton Dickinson
BDIS	Becton Dickinson Immunocytometry Systems
C-PBMCs	Cryopreserved PBMCs
CAB	Community advisory board
CDC	Centres for Disease Control and Prevention
CHER	The Children with HIV early antiretroviral therapy trial
CI	Confidence interval
CIPRA	Comprehensive International Program for Research in AIDS
CMV	Cytomegalovirus
CNS	Central nervous system
CT	Computed tomography
CWC	Child Wellness Clinic
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSMB	Drug safety monitoring board
ELISA	Enzyme-linked immunosorbent assay
FBC	Full blood count

FCS	Fetal calf serum
GMC	Geometric mean concentration
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HEU	HIV exposed uninfected
Hib	*Haemophilus influenzae* type B conjugate vaccination
HIE	Hypoxic ischaemic encephalopathy
HIV	Human immunodeficiency virus (HIV-1 is referred to as HIV in this text)
HLA	Human leucocyte antigen
HREC	Health Research Ethics Committee
ICH	Institute of Child Health
IL-7	Interleukin 7
IMCI	Integrated management of childhood illnesses
KIDCRU	Children's Infectious Disease Clinical Research Unit
KRECs	Kappa-deleting recombination excision circles
LBW	Low birth weight
LRTI	Lower respiratory tract infection
LTNP	Long-term non-progressors
LTR	Long-terminal repeats
MHC	Major histocompatibility complex
NHLS	National Health Laboratory Services
NK	Natural killer cells
OPA	Opsonophagocytic killing activity
PCV7	Pneumococcal conjugate vaccine (7 valent)
PENTA	Paediatric European Network for the Treatment of AIDS
PET	Positron emission tomography
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PHRU	Perinatal HIV Research Unit
PMTCT	Prevention of mother-to-child transmission
PUO	Pyrexia of unknown origin
PUJ	Pelviureteric junction
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
SOP	Standard operating procedure

STI	Structured treatment interruption
SUN	Stellenbosch University
TB	Tuberculosis
TES	Thymic epithelial space
TMB	Tetramethylbenzidine
TCR	T-cell receptor
TRAC	T-cell receptor alpha constant
TREC	T-cell receptor excision circle
UCL	University College London
URTI	Upper respiratory tract infection
UTI	Urinary tract infection
VMI	Visual motor integration
WBA	Whole blood assay
WHO	World Health Organisation

Chapter 1

Introduction

1.1 The Immune System

The immune system protects the body from infectious agents and the damage they cause by four main tasks: immunological recognition, immune effector functions, immune regulation and immunological memory [1]. Immune responses are broadly divided into innate (non-specific) such as physical or chemical barrier and phagocytic responses, or adaptive (specific) responses that are developed during a lifetime as an adaptation following exposure to an organism with the crucial development of immunological memory.

Most infectious agents activate the innate immune system and induce an inflammatory response. This usually commences with secretion of proteins called cytokines and chemokines which essentially initiates the process of inflammation, recruiting the cells and molecules required to attack the pathogen, while increasing the flow of lymph to nearby lymphoid organs and thereby activating the adaptive immune system.

Adaptive immunity is further divided into humoral i.e. production of antibodies, or cellular i.e. mediated through cells of the immune system. Lymphocytes are integral to both pathways, whereby B-lymphocytes (B-cells) produce antibodies and present antigens to T-Lymphocytes (T-cells), and T-cells can act as cytotoxic killing cells or helper cells to recruit or suppress other cells involved in the specific immune response. B-cell development occurs predominantly in the bone marrow but also in the fetal liver, whereas the development of T-cells takes place in the thymus.

The bone marrow

The pluripotent haematopoietic stem cell generates all the cells of the immune system initially committing to either lymphoid or myeloid lineage. These processes typically originate in the bone marrow where a specialized microenvironment provides signals that induce switching on of key genes that direct a developmental program. Non-lymphoid connective tissue stromal cells interact intimately with the developing lymphocytes by interactions between cell-adhesion molecules and their ligands, and also by providing soluble and membrane-bound cytokines and chemokines that control lymphocyte differentiation and proliferation. The lymphocyte progenitor is signaled to give rise to the common lymphocyte progenitor or the early T-lineage precursor, whereby the former differentiates into Pre-B-lymphocytes and Pre-Natural Killer cells, and the latter will migrate from the bone marrow to the thymus and become a thymocyte.

B-cell development

B-cell development occurs in two main phases. An initial antigen-independent phase in the bone-marrow where precursor B-cells mature into functional naïve B-cells and multiple rounds of first heavy- and then light-chain immunoglobulin gene rearrangements occur to generate a diverse range of functional receptors [2]. The progress of this phase of development is dependent on interactions with the bone marrow stromal cells and the cell does not progress to the next phase until a protein chain is produced. The antigen-dependent phase follows whereby mature B-cells are maintained by regeneration, turnover and selection processes. These selection processes include negative selection in the bone marrow whereby self-reactive immature B-cells are removed from the repertoire or die if over-stimulated by antigen. Mature B-cells migrate to peripheral lymphoid organs and are activated when bound to foreign antigen.

B-cells are specialized to recognize the surface antigens on pathogens living outside cells and when activated by these antigens they will differentiate into plasma effector cells that secrete antibodies and long-lived memory cells to target these pathogens. Antibodies are Y-shaped molecules whose arms are composed of two identical heavy and two identical light chains, and form two identical antigen-binding sites. These are highly variable from one molecule to another, however the stem on the Y (constant region) is far less variable and takes five main forms, known as immunoglobulin classes IgA, IgE, IgD, IgG and IgM, which determine the antibody's function. Antibodies participate in host defense in three main ways: neutralization, opsonization and complement activation.

T-cell development

In contrast, T-cell development begins with haematopoietic stem cells, characterized by the presence of CD34 surface antigen, migrating from the bone marrow to the thymus where sequential stages of phenotypic maturation occur. Within the thymic cortex, double negative thymocytes that neither express CD4 nor CD8 cell-surface markers, differentiate into double positive thymocytes expressing both CD4 and CD8. During this time the T-cell receptor (TCR) is assembled through a non-specific process of recombinant rearrangement of two distinct lineages of T-cells from either αβ or γδ genes. The TCR beta chain is formed by rearrangement of the Variable (Vβ), Diversity (Dβ) and Joining (Jβ) regions encoded in the genome with extra diversity acquired though random insertion and deletion of bases between these regions. The rearranged beta chain is then paired with a pseudo alpha chain to establish functional rearrangement before pairing with an actual alpha chain formed by rearrangement of Vα and Dα regions also with insertions and deletions of bases between the regions. Likewise, the γδ chains are formed concurrently in a similar manner, however more than 90% of circulating T-cells express αβ TCRs. These processes produce a desirable diverse range of TCRs to recognize foreign antigens. A by-product of this rearrangement are spliced and excised episomal DNA circles known as T-cell receptor excision circles (TRECs) which have been used as a measure of thymic output.

The subsequent phase of T-cell development occurs in the thymic medulla where thymocytes undergo an extensive selection process to ensure self-tolerance and self-major histocompatibility complex (MHC) restriction eliminating autoreactive T-cells (negative selection) and preserving functional T-cells (positive selection). TCRs

recognise short linear peptides when presented in association with MHC-molecules on the surface of antigen-presenting cells. Only thymocytes whose receptors interact with self-peptides:self-MHC complexes can survive and mature, ensuring T-cells bearing receptors with high affinity for self-peptides that could result in autoimmunity and T-cells with receptors of very low affinity are deleted. At this stage it is also determined which T-cells will become single positive CD4 or CD8 T-cells [3]. These selection processes discard about 99% of all thymocytes and lead to the generation of naïve CD4 and CD8 T-cells frequently referred to as recent thymic emigrants (RTEs) [4] that enter the peripheral circulation.

After leaving the thymus RTEs circulate through the spleen and lymph nodes, and enter a phase of homeostatic cell division. Within the peripheral lymphoid organs the T-cells may encounter their specific antigen and become activated, undergoing clonal expansion and differentiation into effector T-cells [1]. Effector T-cells are attracted to the site of an infection or inflamed tissue and can either kill infected cells or activate macrophages or antibody responses.

The thymus and thymic output

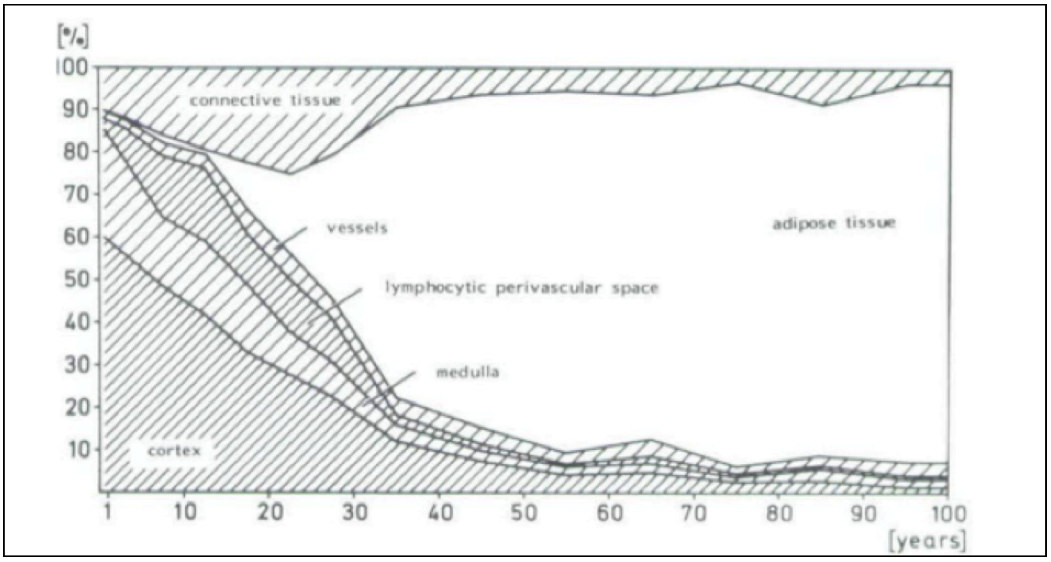
The thymus has no self-renewal properties and relies upon the continuous replenishment of new T-cell progenitors from the bone marrow [5]. Thymic size is maximal at around one year of age and thereafter, uniquely compared to all other organs of the body, the thymus demonstrates an age-associated involution [4, 6, 7]; there is reduction in mass, structure and cellularity, and correspondingly a decline in exported naïve T-cells from the thymus.

Extremely high thymopoesis in the first 2 years of life is believed to correlate with thymic size [8-10]. In line with the involution of thymic epithelial tissue, it has been proposed that thymic export reduces in three phases: from 2 to 5 years, from 5 years to puberty, and thereafter from puberty into adulthood [11]. In adulthood, the T-cell pool is mostly maintained by peripheral proliferation and homeostasis with the thymus having a minimal role, reducing with age, in maintaining immune efficacy.

There are several histological changes associated with aging in the thymus, namely a significant reduction in the volume of the cortex and medulla and replacement with adipose tissue, fibrosis and accumulation of senescent cells. Figure 1.1 presents the age-associated changes in histology from a large morphometric study of autopsied thymuses from 304 adults and children who died of sudden death without infectious

cause [12]. Several other studies have demonstrated that with age, the thymic microenvironment undergoes structural, phenotypical, and architectural changes including down-regulation of various thymic epithelial cell markers [5]. Extrinsic changes associated with ageing such as decreased IL-7, necessary for thymopoiesis, are likely to result in impaired T-cell development.

Figure 1.1: Changes in histological proportions of the thymus with age [12].



Reduction of connective tissue, medulla, cortex and lymphocytic perivascular space is illustrated with proportional increase in adipose tissue.

These changes recognised to occur with ageing and result in decline of thymic output are believed to be associated with reduced T-cell diversity and thereby increased susceptibility to infection, autoimmune disease and cancer [5]. However these associations have not been well described, primarily due to the difficulties in quantifying thymic output since the complex interplay between naïve T-cell production, homeostatic proliferation and apoptosis can mean that peripheral blood sampling of naïve T-cells could be misleading. This association between thymic output, reduced T-cell diversity and increased susceptibility to infection could have particular relevance in children where compromise of thymic output in early childhood years, such as infection with Human Immunodeficiency Virus (HIV) could have crucial implications on their developing immune system and ability to fight infection long-term. The vital role the adaptive immune system in fighting infection is illustrated by diseases of immunodeficiency, and particularly so by HIV, which succeeds in evading suppression of the adaptive immune response.

1.2 The effect of HIV infection on the immune system

The syndrome we now know as Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981 when the first previously healthy patients were found to be suffering from severe immunodeficiency and dying from opportunistic infections. The cause of AIDS was identified in 1983 to be Human Immunodeficiency Virus [13], and 30 years later, 60 million people have been infected with HIV and 25 million have died from AIDS [14]. The first antiretroviral drug was developed in 1986 and by 1996 combination anti-retroviral therapy (ART) had been introduced, simultaneously targeting various stages of the HIV-lifecycle to control viral replication thereby preventing the deleterious impact of HIV upon the immune system and significantly reducing morbidity and mortality.

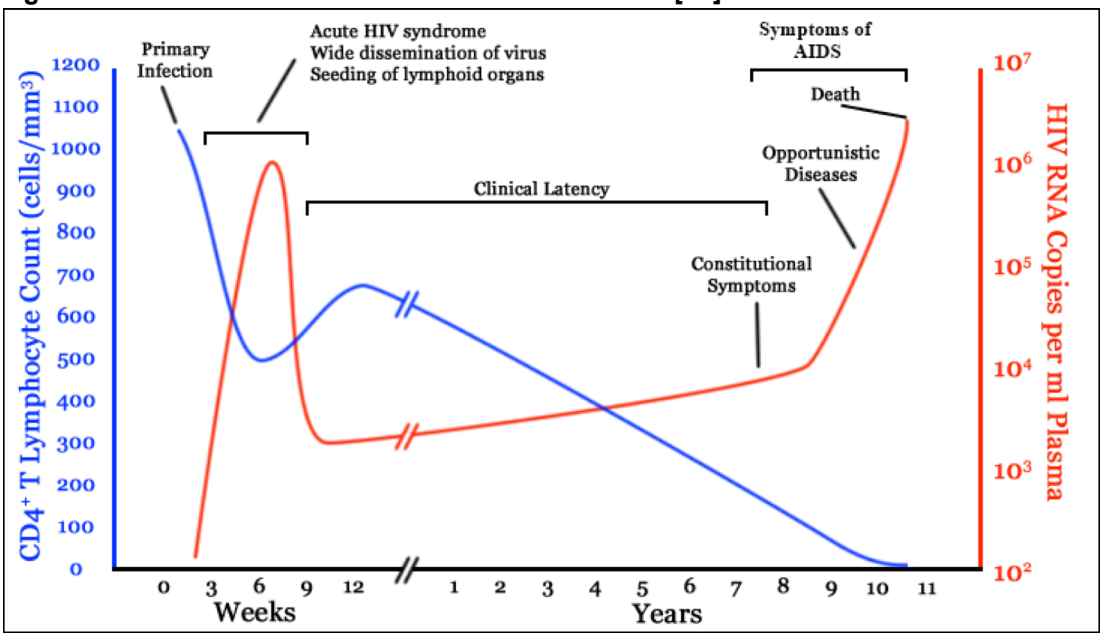
HIV is a retrovirus from the Lentivirus family. Of the two isolates HIV-1 and HIV-2, HIV-1 is globally the commonest form and in this text it is referred to as HIV. There is evidence that HIV originated from Simian Immunodeficiency Virus (SIV) [15] and transferred to humans from chimpanzees and sooty mangabeys [16, 17]. Transmission of HIV occurs through transfer of bodily fluids such as blood, semen, vaginal fluids and breast milk, and infects vital cells of the immune system specifically CD4 T-helper cells, macrophages and dendritic cells.

Three to four weeks following HIV-exposure there is rapid viral replication and development of a flu-like illness. Shortly afterwards, HIV-specific immune responses develop and antibodies to core (p24) and envelope (gp160, gp41, gp120) proteins are detectable in the blood [18]. During this acute phase, there is high viraemia and massive CD4 depletion, the cause of which is incompletely understood [19, 20]. There is evidence for direct viral killing of infected cells, killing of infected CD4 T-cells by CD8 cytotoxic lymphocytes, and immune-activated T-helper cells which undergo repeated rapid mitosis and thereby have increased susceptibility to the induction of apoptosis [4]. In addition, it has been suggested that regeneration of new T-cells may be defective in HIV-infected individuals since progenitor T-cells from within the thymus may be infected and destroyed [1].

Three months following primary infection, CD4 levels partially recover and viraemia subsides, and this depicts the chronic phase, known as clinical latency. Although this phase is often asymptomatic, there is ongoing gradual CD4 T-cell loss [21]. When

CD4 T-cells decline below a critical level, cell-mediated immunity is lost and infections with a variety of opportunistic microbes appear. In the absence of ART, there is progressive immune collapse characterised by frequent opportunistic infections, followed by the development of AIDS and finally death (Figure 1.2).

Figure 1.2: Time course of HIV infection without ART [22].



The first few weeks are typified by an acute influenza-like viral illness with high titres of virus in the blood, (seroconversion). An adaptive immune response follows, controlling the acute illness and largely restoring levels of CD4 T-cells in the blood but does not eradicate the virus. The disease becomes symptomatic with opportunistic infections as the CD4 T-cell count begins to fall. When the CD4 count is <200 the patient has AIDS and death may be imminent without treatment with ART.

HIV enters T-cells through binding of viral glycoprotein envelope (gp120) to the cell surface molecule CD4 and either the co-receptor CCR5 or CXCR4 [23]. Inside the cell, viral RNA is transcribed into double-stranded proviral DNA by viral reverse transcriptase. Within the nucleus the proviral DNA is then integrated into the infected cell's chromosomal DNA where it may remain as a latent reservoir in quiescent cells. However, if the host cell is activated proviral DNA is transcribed back into genomic RNA and is translated into viral proteins (Gag, Env, Gag-Pol) which bud from the infected T-cell and are then released as infectious virus [18]. However subsequent assembly and maturation of virus particles relies upon the activity of the HIV protease enzyme.

HIV has been effective in evading the immune system and numerous antiretroviral drugs for several reasons. HIV replicates at a rate of 10^9-10^{10} new virions each day, with a mutation rate of $3x10^{-5}$ nucleotide per cycle [1] which means that the adaptive immune system cannot generate sufficient HIV-specific memory cells and it is the ideal environment for drug resistance to develop. The HIV protein encoded by Env protrudes through the virion membrane and plays a role in bystander killing of uninfected T-cells [24]; the HIV protein Nef down-regulates the expression of various proteins that are needed for recognition by CD8 T-cells [25]; and the HIV gene Tat appears to up-regulate expression of BCL2 which inhibits cell death [26]. In later stages of the disease, the HIV protein Vpr arrests target cells in the G2 phase of the cell cycle and induces apoptosis in T-cells, peripheral blood lymphocytes and fibroblasts. HIV-specific CD8 T-cells have lower than required levels of perforin to effectively penetrate and destroy infected CD4 T-cells that are producing new HIV virions [27]. In addition, HIV-infection of CD4 memory cells, macrophages and dendritic cells establishes latent reservoirs that have potential to reactivate, release infectious virions and cause further immunocompromise.

Reservoirs for persistence include the central nervous system, gastrointestinal tract and urogenital tract, however a major reservoir of HIV infection is lymphoid tissue, in which infected CD4 T-cells, monocytes, macrophages and dendritic cells are found. Macrophages and dendritic cells appear to harbour replicating HIV without being killed by it, so they are believed to be an important vessel of spreading infection [1] particularly to the brain. HIV is also trapped in the immune complexes on follicular dendritic cells becoming a further source of infective virions.

HIV-infection causes a variety of B-cell dysfunctions [28, 29] which may alter differentially in children of varying ages [30], including decreased proliferative responses [31], loss of memory B-cells, altered differentiation of naïve B-cells and decline in neutralizing antibody responses [32]. This results in reduced quantity and diversity of antigen-specific antibodies [33] which could compromise virological control. The mutations that occur as HIV replicates allow the resulting virus variants to escape recognition by neutralizing antibody or cytotoxic T-cells and contribute to the long-term failure of the immune system to contain the infection.

The use of combined ART in targeting several phases of HIV life cycle simultaneously has been effective in avoiding evolving drug resistance. Such antiretroviral pharmaceuticals include fusion inhibitors, nucleoside and non-

nucleoside reverse-transcriptase inhibitors, integrase inhibitors, and HIV-protease inhibitors [34]. ART reduces HIV-RNA replication and its associated CD4 T-cell destruction allowing immune reconstitution to follow with an increase in CD4 count [35]. However, the degree with which immune restoration occurs varies among patients and despite effective control of viral replication, some adult patients do not achieve a sufficient immunological recovery [36]. It is possible that differences in CD4 reconstitution may, in part, be due to variances in supply of naïve T-cells [7]. Thymic output along with homeostatic cell division is essential for the generation of naïve CD4 and CD8 T-cells and is especially important in early childhood years during the development of the child's immune system. There is data to support the contribution of thymic output to immune-recovery in HIV-infected adults on ART [37] and it is therefore likely to play an important role in children.

The main immunological effect of HIV infection is the destruction of CD4 T-cells that occurs through the direct cytopathic effects of HIV and through killing by CD8 cytotoxic T-cells. HIV establishes a state of persistent infection in which the virus is continually replicating in newly infected cells. HIV combines the characteristics of a persistent infection with the ability to create immunodeficiency in its host and illustrates the importance of innate and adaptive immunity in an effective immune response against infection. The key to fighting pathogens such as HIV is the use of immunological research to develop our understanding of the immune system and its role in combating infection.

1.3 HIV infection in infants and children

Currently, an estimated 34 million people are living with HIV worldwide, including 3.4 million children and adolescents [38]. With effective prevention of mother-to-child transmission (pMTCT) new infant infections have been declining, however there are still up to 450,000 new infant infections each year [39]. In South Africa, maternal-to-infant HIV-exposure is now 32% with a transmission rate of 3.5% [40]. For the infants that are infected with HIV, it is vital that treatment strategies minimise disease adverse effects and treatment side effects, and ultimately optimize the development of a functional immune system that will protect them throughout their lives.

Numerous challenges exist in the treatment of infants and young children infected with HIV and these are magnified in resource-limited settings where the majority of the HIV-infected children reside. Due to healthcare infrastructure, availability of resources and the fact that virological testing was required to confirm HIV-infection status, identification of infected infants may have been delayed [41]. This is a significant obstacle since disease progression can be rapid, with peak mortality in the first few months of life [42], yet there are currently no reliable markers to predict rapid disease progression [43]. There is a limited range of antiretroviral drugs available for children especially in affordable, palatable and appropriate formulations. Moreover, dosing must be regularly readjusted in line with infant and child growth. Across childhood, but particularly in adolescents, continuous adherence to ART is often a huge challenge. Non-compliance may result in drug-resistance and thereby compromised clinical management from poor availability of second or third-line ART. There may also be potential metabolic, cardiovascular and neurological problems from long-term ART started early in life.

Current international guidelines recommend commencing ART for children less than 5 years of age [44] with strong evidence from the Children with HIV Early Antiretroviral Therapy (CHER) Trial that supports ART initiation before 1 year of age [45]. However, minimal evidence and guidance exists for how long to continue ART. The implications of ongoing ART are not insignificant in terms of compliance, drug-resistance, drug-related side-effects, toxicity, and finance [46]. Continuous monitoring of these life-long medications are required to understand the multiple possible effects on the developing child. One such effect upon the immune system of ART started within months of birth and subsequent effective viral suppression is the increasing recognition of HIV-antibody seronegativity [47-51]. Therefore, some of the important clinical questions for management of HIV-infected infants and children are: how long should ART be continued in childhood, whether treatment interruption is safe, and if so, when, for whom, and for how long? To begin to answer these questions, further more detailed understanding of the immunology and virology of paediatric HIV-infection is required.

Perinatally acquired HIV may be transmitted from the mother in-utero, intrapartum or postpartum [52]. In view of the developing status of the immune system, the timing of infection might be an important determinant of outcome. The known effects of HIV on the immature immune system include limited production of cytokines, delayed cytotoxic lymphocyte activity, thymic aplasia and early depletion of naïve T-cells [53-

56]. These factors, along with the size of the thymus and CD4 pool, may contribute to the high levels of viral replication that is characteristically seen in the newborn, the longer time required to decline to viral set-points [57-59] and the rapid disease progression with increased mortality that is seen in infants [60, 61]. Following the initiation of ART, the capacity of the thymus to restore the naïve CD4 pool after HIV-induced depletion is likely to be crucial in children, particularly in infancy since a competent peripheral circulating TCR repertoire may not have been established.

1.4 The role of thymic output in immune reconstitution

The rate of CD4 loss with HIV infection, and the degree of restoration with ART, is determined by the complex interplay between rates of HIV-induced T-cell death, peripheral T-cell proliferation and thymic production of new T-cells [21, 62]. In view of the high thymic activity in young children, the export of new T-cells from the thymus is likely to have particular importance in HIV-infected children. In whom the aim of ART should be to preserve the child's developing immune system and maximize successful immune reconstitution.

Despite age-associated decline of thymic function, there does appear to be a role of the thymus in immune reconstitution in HIV-infected adults. Larger thymic size has been associated with improved recovery of total and naïve CD4 T-cells, increased TREC frequencies, and renewal of TCR repertoire diversity [37, 63]. CD4 T-cell restoration appears to be most successful within two years of infection, when it is likely that the thymus is more productive. In addition to chronic immune activation and ongoing viral replication in untreated HIV-infected individual, reduced thymic output might also, in part, explain the large variation in CD4 recovery after ART is initiated [64]. This is supported by surrogate measures such as naïve CD4 T-cell counts and percentage of recent thymic emigrants (CD4+CD45RA+CD31+ lymphocytes) that appear to predict potential for immune reconstitution [36, 65], however these analyses do not account for varying rates of peripheral cell division or death.

A recent study of 60 HIV-infected children and adolescents suggested that age-adjusted thymic output is reduced in untreated HIV-infected children but significantly increases with ART [66] and suggests that after starting ART, the thymus is able to at

least partly compensate for the loss of naïve CD4 T-cells by increasing thymic output. This finding reinforces the importance to further investigate the role of thymic output in immune reconstitution in HIV infection and how ART-strategies may be able to optimize immunity in HIV-infected children.

1.5 Aims and structure of study

As this chapter describes, in HIV-infected individuals CD4 T-cell loss results in profound immunodeficiency and susceptibility to infection, however loss of CD4 T-cells can be partially reversed by ART. The benefit of early ART in terms of reduced morbidity and mortality is widely recognized, however there remain many unanswered questions regarding the influence of HIV and early ART on the child's developing immune system, including thymic output, naïve B-cell output, HIV-antibody responses, and HIV proviral DNA reservoirs.

Increased understanding of the mechanisms by which HIV and ART affect the immune system of a child could help to address issues in ART management strategies for HIV-infected children such as how long to continue ART in childhood, and whether to have ART interruptions and if so, when. However, a detailed understanding of the paediatric immune system in healthy children from within comparable populations is prerequisite (Chapter 3). With these data, comparison between healthy HIV-uninfected children and HIV-infected children is therefore possible to characterize the immunophenotypic response to HIV and ART (Chapter 4).

In view of the evidence that supports the contribution of thymic output to immune reconstitution [37], and to the preservation of a diverse and functional T-cell repertoire, it is necessary to understand the relationship between thymic output and HIV, with and without ART (Chapter 5). This understanding is likely to be enhanced by appreciating the potential relationship between naïve B-cell output and thymic output and the influence of HIV and ART on naïve B-cell output (Chapter 6). Current issues such as poor reactivity of HIV-antibody assays in children on ART from infancy (Chapter 7) and optimising strategies to reduce the peripheral HIV proviral DNA reservoir have also been examined (Chapter 8).

Specific aims:

Immune parameters in healthy South African children:

- To establish local reference ranges for haematological and immunological parameters of healthy South African children.
- To describe differences between immunological markers in children from low and high disease-burdened settings.
- To explore the effect of cryopreservation on immunological parameters.

Immunological responses to HIV and ART in children:

- To compare immunological phenotypes of healthy HIV-uninfected children with HIV-infected children on or off ART.

The influence of HIV and ART on thymic output in children:

- To describe normal thymic output in healthy South African children.
- To examine the impact of the CHER ART strategies on thymic output.
- To explore the relationship between thymic output and HIV disease progression.

Naïve B-cell output in healthy and HIV-infected children:

- To define reference ranges for KRECs and naïve B-cell output in healthy children.
- To examine the impact of the CHER ART strategies on naïve B-cell output.
- To describe the relationship between thymic output and naïve B-cell output.

HIV-seronegativity with early ART:

- To compare the effect of early versus deferred ART on HIV-antibody serostatus.

HIV proviral DNA reservoirs and early ART:

- To examine the effect of the CHER ART-strategies on peripheral reservoirs of HIV.
- To explore the relationship between HIV serostatus and HIV proviral DNA.

The laboratory techniques for this study were learned at the Institute of Child Health (ICH), University College London (UCL) during the first 6 months of this fellowship. Two subsequent years were spent in South Africa collecting samples and performing the laboratory work at Stellenbosch University (SUN) immunology laboratory. The Child Wellness Clinic (CWC), a paediatric health promotion clinic in an informal settlement, was established to acquire healthy control participants. Peripheral blood

mononuclear cells (PBMCs) and plasma specimens from HIV-infected children were taken from “The Children with HIV Early Antiretroviral Therapy (CHER) Trial”. Frequent visits were made to the two sites of the CHER trial: The Perinatal HIV Research Unit (PHRU), Witwatersrand University, Johannesburg and The Children’s Infectious Disease Clinical Research Unit (KIDCRU), Stellenbosch University, Cape Town to collaborate with the protocol co-chairs of the CHER trial throughout my fellowship.

Chapter 2

Methods and Study Populations

This chapter describes the two populations used in this fellowship: HIV-uninfected healthy South African children from “The Child Wellness Clinic” (CWC) and HIV-infected children from the “Children with HIV Early Antiretroviral Therapy (CHER) Trial”. Also described are the specimens, laboratory techniques and statistical approaches applied for the six areas of analysis: immunological phenotyping in health and HIV-infection, thymic and naïve B-cell output, HIV-antibody and HIV proviral DNA quantification.

2.1 The Child Wellness Clinic

A “Child Wellness clinic” for children from birth up to 13 years of age was established at a community health clinic in the informal settlement of Wesbank, Eastern subdistrict of Cape Town. The primary aim of the CWC was the acquisition of HIV-uninfected blood specimens to measure “normal” thymic output and to create haematological and immunological reference ranges in healthy children from South Africa. The clinic was also designed to benefit the participants and the wider community in terms of health promotion, education and screening. Sampling from

this population aimed to obtain a group of HIV-uninfected children, comparable to CHER participants in terms of socio-economic status, nutrition, vaccination and environmental exposure to infectious pathogens.

Within the region of Wesbank 27% are unemployed, 62% of adults did not complete education to equivalent of GCSE level, and 39% of households earn below (R19,200 per year = £1,422). 16% live in informal dwellings and only 5% of the population is above 65 years of age. The infant mortality rate for Wesbank is 23.2 deaths per 1000 live births, 7% of babies are born to mothers aged under 18 years, and 20% of babies are born weighing less than 2.5kg [67].

CWC attendance was voluntary but the criteria for recruitment was that the child was well at the time with no chronic medical condition, registered at Wesbank health clinic, attended with their biological mother and brought their "Road to health" (hand-held child medical record) card with them. After informed consent was obtained in English or via translator in Afrikaans or Xhosa, the clinic session included clinical history and examination by a paediatrician, anthropometry plotted on road to health card growth charts, assessment of vaccination status with catch-up if necessary, provision of nutritional supplements (vitamin-enriched porridge and peanut butter) and a supermarket food voucher. Each participant had phlebotomy of 2-3mls of blood that was used for rapid HIV-antibody analysis, full blood count (FBC), basic immunological profile and thymic output.

If the rapid HIV-antibody tested positive in a child less than 18 months of age, provision was made for blood to be sent to the local laboratory for HIV proviral DNA and for mother and child to be referred to the local family HIV service (unless already known). Any child found to be HIV-infected would not be included in the study. Children found to be HIV-exposed but not infected (HEU) were not included in the healthy control population, however thymic output was still measured from these children and used for subgroup analysis. Since a child older than 18 months with a negative HIV-antibody test may have been HIV-exposed, maternal HIV-status was also assessed verbally, by reviewing the child and mother's clinic files and by accessing the National Health Laboratory Service (NHLS) database with the maternal facility number.

The clinic ran for 2 mornings per week for 8 months. From a total of 487 recruited, 15 did not meet criteria or it was not possible to obtain enough blood for research

purposes and 1 mother withdrew consent, therefore 471 met criteria and a blood test was acquired for research purposes. Although the CWC was initially designed to recruit children less than 2 years of age, the clinic was extremely popular and therefore, after 4 months, the CWC was extended to all children under the age of 5 years, and for all children up to 13 years of age during school holidays.

Appendices I – IV detail the following documents for the CWC: poster and flyers, information and consent form (which were also translated into Afrikaans and Xhosa), structure of the clinic session and CWC proforma completed for each participant. Appendix V details the components and definitions of information that were retrieved, coded and anonymously recorded from the proforma. When completed the proforma, consent form and blood test results were filed in the child's medical notes at Wesbank clinic.

Ethical considerations and public engagement of the CWC

The recruitment of healthy controls for this study was registered as a clinical trial with ethics approved by the Health Research Ethics Committee (HREC) at Stellenbosch University. It was also approved by the CIPRA-SA committee, the Head of the Department of Paediatrics at Stellenbosch University, the Head of Community Division of Paediatrics, Tygerberg Hospital Managers Committee and the City of Cape Town Department of Health (Appendix VI: Ethical and Regulatory Approvals).

The CWC participant information sheet was provided in the child's guardian's first language and a signed consent form was obtained from a guardian of each child participant via a translator in Afrikaans or Xhosa if required. One copy of the consent form was given to the guardian, one copy kept with the participants clinic file and a third kept in locked files at KIDCRU as part of quality control and governance. To maintain subject confidentiality each participant was given a study registration number and all data were anonymised and transcribed onto an encrypted electronic database. The blood sample for thymic output was coded with the study registration number and date of birth only, and the sample sent for FBC had initials and date of birth as patient identifiers. Full name and contact details were only retained at Wesbank clinic and all documents containing patient identifiers were kept in locked cabinets in secure locations.

Every CWC participant had an HIV-antibody test performed. Since a positive test may reflect the mother's HIV-status, counseling was given to the mother during the

consent-taking process. If any child were to be found HIV-antibody positive, every effort would be made to ensure that they attend their follow-up appointment with the local family HIV service while maintaining patient confidentiality. The risk of bruising and potential discomfort experienced during phlebotomy was explained to the parents and child. For those running the CWC, potential risks included the handling of HIV-infected blood. Universal precautions were applied during the taking of all samples and during laboratory processing of blood. Gloves were worn when collecting or handling blood, and goggles when there was danger of blood splashing. All needles and sharp objects were disposed in puncture-resistant containers.

Aside from acquiring blood specimens from healthy children to establish local reference ranges and explore thymic output, the CWC had a number of concurrent benefits including health promotion, optimisation of vaccination, increased HIV-screening in high risk groups, and public engagement with health research. It transpired that a number of children attended the CWC had not been accessing routine paediatric care, therefore attending the CWC was an opportunity to catch-up on missed vaccinations, de-worming and vitamin A drops as well as to reinforce the importance of existing public health strategies.

Good community relations are vitally important for the success of clinical research and KIDCRU has an extensive history of community projects with benefits to children. Regular meetings regarding this study were held with the Community Advisory Board (CAB), a group of people that aim to make a difference in the lives of HIV-infected people, caregivers and the research team. The KIDCRU CAB consists of parents, caregivers, legal guardians of HIV-infected children, service-providers, medical caregivers, and interested community members. The CAB functions to provide input from the community to the research team and to be the advocate between the research participant and the research team to facilitate informed decision-making. These individuals were best suited to receive information regarding the CWC study, progress updates and subsequently transmit the relevant findings to the community and participants of the study. Results were also presented and displayed in poster form in Wesbank community health clinic and Tygerberg Hospital.

Every effort was made to ensure that my presence, as a paediatrician, at Wesbank clinic would be helpful and would not undermine nor hinder the work of the clinic staff. Positive benefits have included increasing the paediatric skill base of the clinic assistant and translator, promotion of the profile of KIDCRU CAB and following the end of the CWC Wesbank community clinic continues to be used for further clinical research recruitment related to other KIDCRU studies.

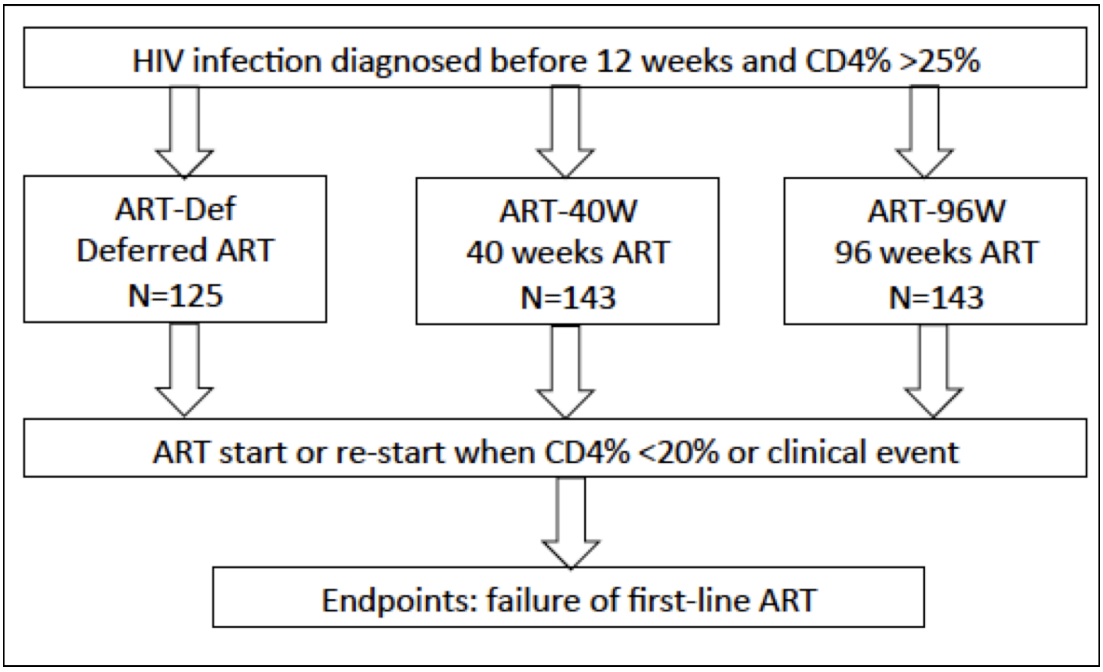
2.2 The Children with HIV Early Antiretroviral Therapy (CHER) Trial

The CHER trial was a phase 3, randomized 3-arm trial conducted by the Comprehensive International Program for Research in AIDS (CIPRA). The CHER trial took place, and post-trial follow-up continues, at the Perinatal HIV Research Unit (PHRU), Johannesburg and the Children's Infectious Diseases Clinical Research Unit (KIDCRU), Cape Town. The trial's main hypothesis was that early limited ART started within 12 weeks of life, compared with deferred ART until clinical or immunological deterioration, would prevent disease progression and safely allow a subsequent period off ART.

377 HIV-infected asymptomatic infants less than 12 weeks of age (median age 7.3 weeks [IQR: 6.4-9.0]) were randomised to three arms: deferred ART until clinical or immunological criteria were met (Arm 1: ART-Def); and early ART for 40 or 96 weeks (Arm 2: ART-40W and Arm 3: ART-96W) followed by a period of ART interruption until clinical or immunological deterioration (Figure 2.1). The trial defined endpoint was time to death or failure of first-line ART, defined as CD4% <20%, CDC stage B/C events or regimen-limiting drug-related toxicity. Criteria for starting or re-starting ART were CD4% <20% or CDC stage C or severe B, i.e. moderately or severely symptomatic with clinical conditions such as serious bacterial infections, encephalopathy or wasting syndrome. This was revised to CD4% <25% or <1000cells/µl in 2006 with international guidelines. First-line ART comprised zidovudine, lamivudine and lopinavir-ritonavir; and second-line ART was didanosine, abacavir and nevirapine or efavirenz. CHER participants were reviewed 4-weekly until week 24, 8-weekly until week 48, and then 12-weekly thereafter. Blood tests done at each review were: full blood count, aminotransferase levels and CD4 count.

In 2007, after a median follow-up of 40 weeks (IQR: 24-58), interim data showed that 66% of infants in the deferred group had started ART. A 76% reduction in risk of death and 75% reduction in disease progression was observed with early ART (both ART-40W and ART-96W combined) compared to deferred ART (p=0.0002) [45]. As advised by the Drug Safety and Monitoring Board, infants randomised to defer ART were then reassessed by their clinicians to commence ART. Early ART initiation for all infants subsequently became the international standard of care, regardless of CDC staging or CD4 count/percentage. Almost all children from ART-Def commenced ART and were followed up as planned. To sufficiently power further analysis of the other randomised arms, ART-40W and ART-96W, 17 more children were recruited to each arm.

Figure 2.1: Overview of CHER trial randomisation and schedule.

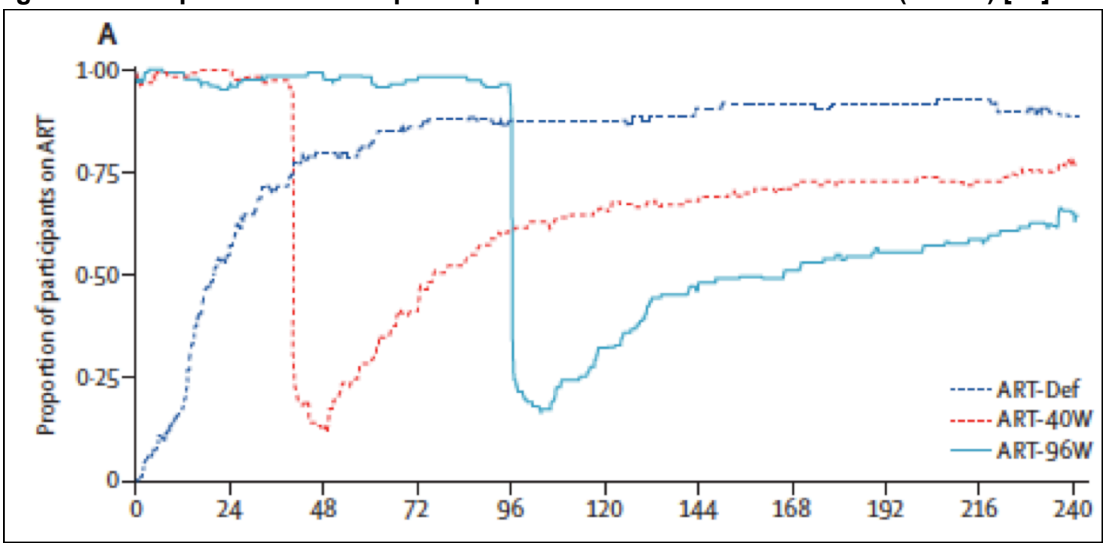


First-line ART was Zidovudine, Lamivudine and Lopinavir. Initially 126 children were recruited to each of the early ART treatment arms (ART-40W and ART-96W) and 125 children to ART-Def (377 in total). However, following the discontinuation of ART-Def in 2007 due to the 75% increased risk of disease progression, ART-40W and ART-96W each had 17 more children recruited to sufficiently power further analysis.

At the trial end in June 2011, 341 of the original 377 children recruited (91%) had either completed follow-up or died (42/377, 11%) over a median duration of 250 weeks (range 182-307 weeks). 34 (9%) were lost to follow-up and 2 (0.5%) withdrew consent. The deferred group received continuous ART (ART-Def) from median 20 weeks (95% Confidence Interval (CI): 16-25). ART-40W received early limited ART,

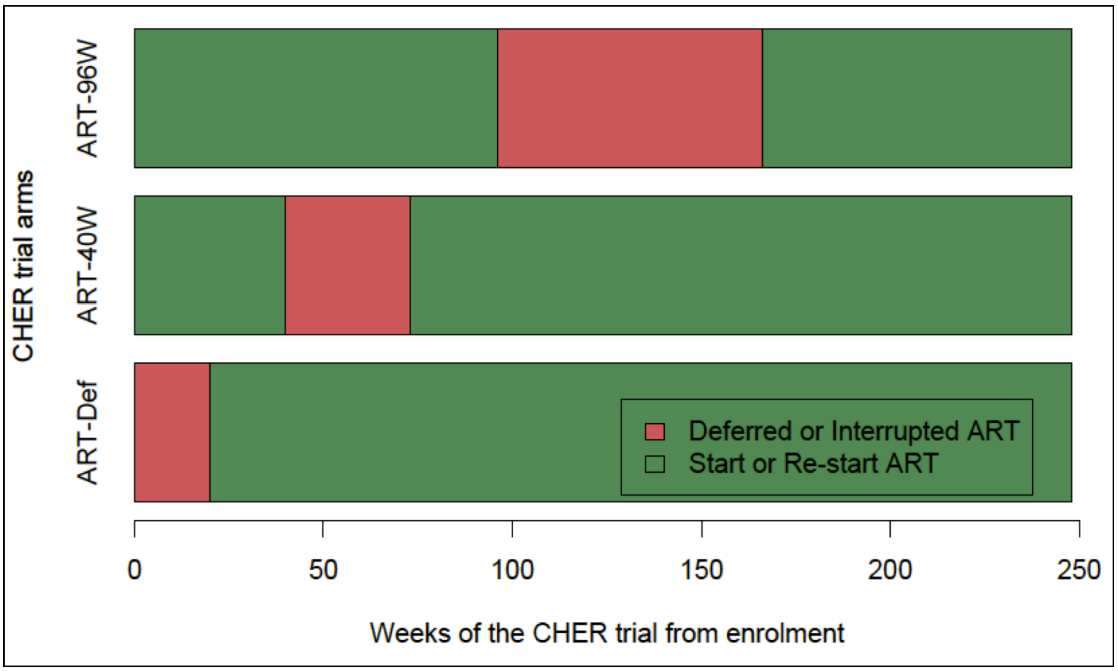
interrupted after 40 weeks and restarted ART after a median of 33 weeks (IQR: 26-45). ART-96W received early limited ART, interrupted after 96 weeks and restarted after median 70 weeks (IQR: 35–109) (Figure 2.2 and more simplistically represented in Figure 2.3).

Figure 2.2: Proportion of CHER participants on ART over calendar time (weeks) [68].



The rate of starting ART: ART-Def (dark blue), ART-40W (red), ART-96W (light blue).

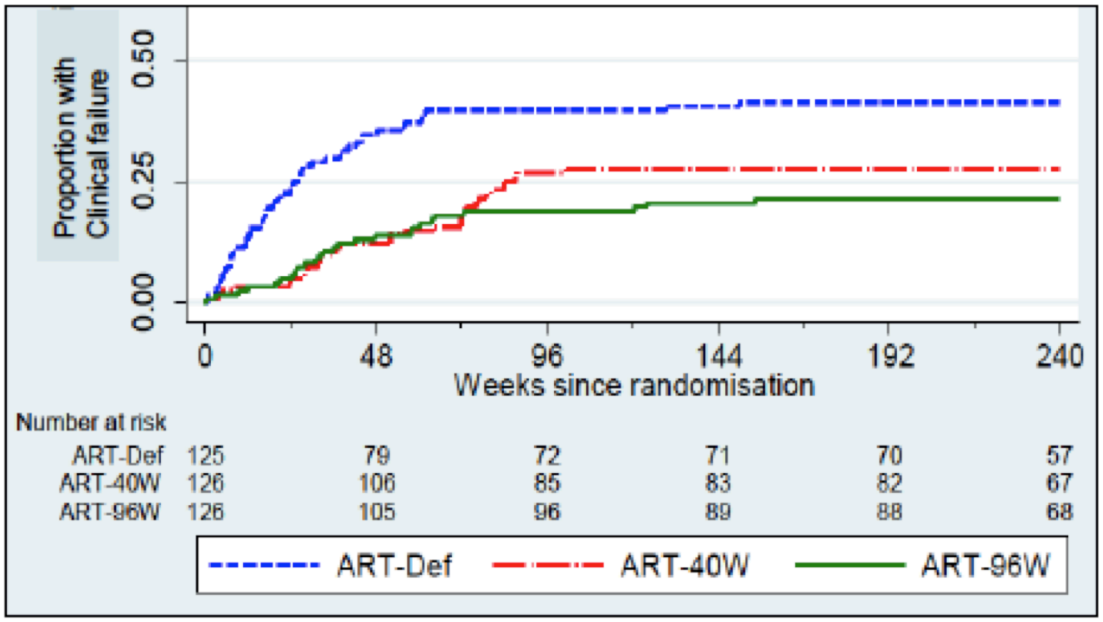
Figure 2.3: Overview of CHER trial treatment strategies and median duration on ART within arms.



Median time to ART initiation in the deferred arm was 20 weeks (IQR 16-25); ART was otherwise started at age 7.3 weeks (IQR: 6.4-9.0). Median duration of ART-interruption for ART-40W was 33 (26-45) and 70 (35-109) weeks in ART-96W. Key: median time of deferred or interrupted ART (red), median duration of time on ART (green).

There were 48/125 (38.4%) children who met trial defined endpoints in ART-Def, including 21 (16.8%) deaths. Twenty-seven (21.6%) endpoints occurred while on ART (10 virological, 9 immunological and 8 clinical failures). In the early limited treatment groups, ART-40W and ART-96W, 32/126 (25.4%) and 26/126 (20.6%) endpoints were reached respectively, including 11 (8.7%) and 9 (7.1%) deaths, and endpoints that occurred while on ART were 21 (16.7%) and 17 (13.5%). There were no failures due to regimen-limiting toxicity. The hazard ratio for progression to severe CDC B/C or death in children with early limited ART compared to deferring ART was significantly reduced for both ART-40W (0.5 95% CI: 0.3 – 0.8; p=0.005) and ART-96W (0.4 95% CI: 0.3 – 0.7; p=0.0003 [68]) as Figure 2.4 illustrates. This figure also suggests that there is no difference in progression rates between ART-40W and ART-96W from Week 40 to 72 when ART-40W interrupts compared to ART-96W continuing ART. However, following this period the rate of progression continued to increase in ART-40W above the rate of ART-96W.

Figure 2.4: Progression to severe CDC B/C or death in CHER [68].



Median time to ART initiation in ART-Def was 20 weeks (IQR 16-25). Median duration of ART-interruption for ART-40W was 33 (26-45) and 70 (35-109) weeks in ART-96W.

By the trial end at 248 weeks, median CD4% was above 30% in all arms, anthropometric data were within population standards and comparable between arms, and only 26 (6.9%) children on ART had a viral load >1,000 copies/ml of blood. Twenty-four (19%) and 40 (32%) of children in ART-40W and ART-96W respectively had not restarted ART and were clinically well. The CHER database does not contain patient identifiers and information stored is protected by confidentiality

procedures approved and regulated by the HRECs of Stellenbosch and Witwatersrand Universities.

Neurocognitive data

At 2 and 5 years of age, the CHER participants at KIDCRU underwent a comprehensive Griffith's neurodevelopmental assessment as part of a neurodevelopmental sub-study of CHER lead by Dr Barbara Laughton. The framework of the assessment included locomotor, language, eye-hand coordination, performance and practical reasoning. These data were made available for analysis alongside HIV proviral DNA estimates of the latent reservoir performed from samples taken at 96 and 248 weeks (approximately 2 and 5 years of age) to determine whether peripheral levels of proviral DNA from blood, might reflect levels in the CNS that may have had an impact on neurological development.

Cytomegalovirus data

Plasma from enrolment of CHER was used to assess CMV DNA-status and quantify CMV (copies/ml). Marvin Hsiao at the National Health Laboratory Service (NHLS) in Tygerberg, Cape Town performed these assays and the data were made available to explore the relationship between CMV and levels of HIV proviral DNA.

Functional antibody responses to routine immunisations

Sixty-six percent of children from CHER participated in CIPRA-4, a parallel study lead by Professor Shabir Madhi which examined the affect of HIV and ART on quantitative and qualitative antibody responses to pneumococcal (PCV7) and *Haemophilus influenzae* type B (Hib) conjugate vaccinations in infants [69]. In South Africa PCV7 is given at 6 and 14 weeks followed by 9 months of age, and Hib at 6, 10 and 14 weeks followed by 18 months as part of the 5-in-1 vaccination alongside diphtheria, tetanus, pertussis and polio [70]. Antibody responses in the form of geometric mean concentrations (GMC) for the 7 serotypes of the PCV7 (4, 6b, 9v, 14, 18c, 19f and 23f) and Hib were measured at 10, 14 and 18 weeks and again at 2 years of age in CIPRA-4. Opsonophagocytic killing activity for serotypes 23f, 9v and 19f and serum bactericidal assay were also measured. The relationship between these antibody responses after vaccination in infancy (n=30) and at 2 years (n=51) and naïve B-cell output estimates was explored within individual CHER participants.

2.3 Specimen preparation

2.3.1 The Child Wellness Clinic specimens

487 healthy infants and children were recruited from the CWC, from whom 471 blood specimens were obtained. The blood was used for immediate HIV-antibody analysis using Alere Determine® rapid tests, 500µl was sent for FBC analysis by the commercial laboratory BARC, 500µl was sent to NHLS in at Witwatersrand University Johannesburg for immunological profile by fresh whole blood assay (WBA), and the remaining blood (1-2ml) was cryopreserved at Stellenbosch University (SUN) Immunology laboratory for thymic output and immunological profile.

Blood samples were taken between 9am and 1pm and stored in EDTA tubes at room temperature. If less than 3mls of blood was obtained, the tests were prioritised in the following order: (1) rapid HIV-antibody test, (2) thymic output, (3) FBC and (4) immune profile (section 2.4.2). There were a few weeks during the CWC when Witwatersrand laboratory was unable to receive samples, and it was not possible to obtain blood from 16 participants, therefore, of the 487 CWC attendees the following samples were obtained: 471 research samples, 425 FBCs and 381 immune profiles.

Of the 471 research samples obtained, 1 child was excluded for severe malnutrition and 16 samples were not used for technical issues. These issues were: 4 freezer vials became stuck and broke in the freezer box, 3 samples were lysed from excess red cell lysis buffer, no Ki67 values were measured in 3 samples due to incorrect intracellular staining, and 5 PCR runs produced unusable TREC results (either no TRECs, no TRACs or high TREC/TRAC ratio resulting in negative thymic output due to mathematical constraints of the thymic output formula). Therefore 454 samples were used to explore thymic output in healthy South African children, and after excluding 19 HIV-exposed uninfected children, 435 samples were used to compare with measures of thymic output from HIV-infected children from the CHER trial.

The data from the 425 FBCs and produced from the whole blood assay done on 381 samples were used to establish haematological and immunological reference intervals for South African infants and children. The data were also compared to the same immunological parameters produced from the Cryopreserved-PBMCs (C-PBMCs) in each child to identify differences in techniques and the impact of cryopreservation on the immunophenotypes examined.

2.3.2 The CHER trial specimens

The routine blood tests that were performed at each clinical review during the trial were: haematology, biochemistry and immunology, and if consented, cryopreserved plasma and PBMCs for research purposes. A total of 5-10mls were taken at each review and participant refusal of consent for storage was <5%. For the first 24 weeks of the trial, the participants had 4 weekly clinical reviews, followed by 8 weekly reviews for 24 weeks, and 12 weekly thereafter.

Up to 40 samples of cryopreserved PBMCs were randomly selected for each of the time-points required to fulfill the original objective of this fellowship: to examine the affect of HIV and ART on thymic output (see power calculation in section 2.5). At each time-point two-thirds of the samples were from PHRU and one-third from KIDCRU to accurately represent the proportion of patients from each centre. Samples were excluded if insufficient PBMCs were stored (i.e. $<10^6$ PBMCs), if they did not adhere to the treatment strategy of their randomisation arm, or if the stored specimen was acquired more than 6 weeks from the intended storage visit date. Due to these criteria and sample availability, in certain comparison groups less than 30 samples were obtained. Exact numbers of specimens and their time-points are illustrated in Table 2.1 and 2.2.

Table 2.1: Specimen numbers processed for thymic output and immunophenotypes at specific time-points of the CHER trial.

CHER trial week	0	4	12	40	60	96	152	248	330-430
ART-Def	46					39			11
ART-40W	44		2		34	47	9	41	20
ART-96W	48	30	28	33	36	45	19	36	13

Table 2.2: PBMC specimens processed for HIV proviral DNA at specific time-points of the CHER trial. NB. There were some samples that were used for all three analyses: thymic output, immunophenotyping and proviral DNA.

CHER trial week	40	96	156	204	248	252
ART-Def	0	45	14	13	75	5
ART-40W	0	0	0	0	56	0
ART-96W	9	73	0	0	44	0

Applications were made to both the HRECs of Stellenbosch and Witwatersrand Universities to waive consent for the use of the stored specimens to measure thymic output (Appendix VI: Ethics Approvals). This decision was made in view of minimal

risk of harm to the participants and as prior consent for storage and HIV-related immunological (non-genetic) research had already been given.

The stored plasma from CHER were used for viral load analysis retrospectively since at the time of the trial viral load measurements were not standard of care. Funding has since become available however, during the analysis phase of this fellowship the dataset of viral loads was not fully complete especially during ART interruption.

Although the CHER trial officially ended in June 2011, 75 CHER participants from KIDCRU were also enrolled in a neurological follow-up cohort (CHER PLUS). These include 20 from ART-Def, 31 from ART-40W and 24 from ART-96W. PBMCs were cryopreserved from yearly routine blood tests in this cohort and used to measure thymic output between 7 and 8 years of age (Weeks 330-430, Table 2.1), thereby capturing a longitudinal perspective of the affect of different ART-strategies used by CHER on thymic output (Appendices VII – VIII: Ethical Approval and patient information/consent forms).

A small number of samples were used from HIV-infected children from a London clinic to establish and optimize the thymic output assays. Ethical approval through IRAS was not required for this purpose since there is existing ethical approval within the immunology department at ICH UCL that left-over blood from routine blood tests may be used for assay-optimisation.

2.4 Laboratory techniques

2.4.1 PBMC isolation and cryopreservation

All patient specimens of PBMCs that have been used in this study have been cryopreserved (CHER and CWC). PBMC specimens have been isolated and cryopreserved according to Clinical Laboratory Services protocol for CHER samples and according to ICH UCL protocol for CWC samples and CHER PLUS samples.

PBMCs were isolated from fresh blood by Lymphoprep gradient separation and cryopreserved in 10% Dimethyl sulfoxide (DMSO) and 90% Fetal Calf Serum (FCS) at a ratio of between 7-15 x 10^6 cells/ml of freezing media for CWC specimens, and CHER specimens between 5-10 x 10^6 cells/ml. CWC and CHER PLUS samples were placed in a Thermo Scientific Nalgene freezing container (Mr Frosty®) for 24-48 hours at -80°C, then at -80°C for 6 months, and transferred to liquid nitrogen thereafter (Appendix IX: PBMC isolation SOP). CHER specimens date from July 2005 and have been kept in liquid nitrogen until used.

Specimens were removed from liquid nitrogen or -80°C when required, thawed quickly in a water-bath at 37°C, removed from the cryovial and gently resuspended in 60% FCS and 40% RPMI at 37°C. The cells were then washed twice in RMPI with 2% FCS to remove traces of DMSO, counted and divided for flowcytometry (minimum 10^5 cells per panel), naïve CD4 T-cell isolation using magnetic columns and where enough cells were available, for quantification of kappa-deleting recombination excision circles (KRECs) from PBMCs (Appendices X-XI: SOPs for Thymic Output and for TREC/KREC quantification).

2.4.2 Immunophenotyping of cryopreserved PBMCs

Healthy infants and children had two panels for analysis (panels A and B below):

- Panel A: viability marker, CD3, CD4, CD8, CD45RA, HLA-DR, Ki67
- Panel B: viability marker, CD3, CD56, CD19, CD27, IgD, Ki67

Of the 471 stored research samples, 461 children had Panel A and 444 children had Panel B (10 samples were unusable as described in section 2.3, and 17 samples had insufficient cells for both panel A and B). Surface staining was performed on thawed PBMCs using anti-human fluorochrome-conjugated antibodies and fixable viability

dye eflour®660 (Invitrogen, BioLegend or eBioscience) for 30 minutes at 4°C before fixation and permeabilsation with FOXP3 fixation/permeabilsation kit (eBioscience). Intracellular staining with Ki67 was subsequently performed for 30 minutes at 4°C.

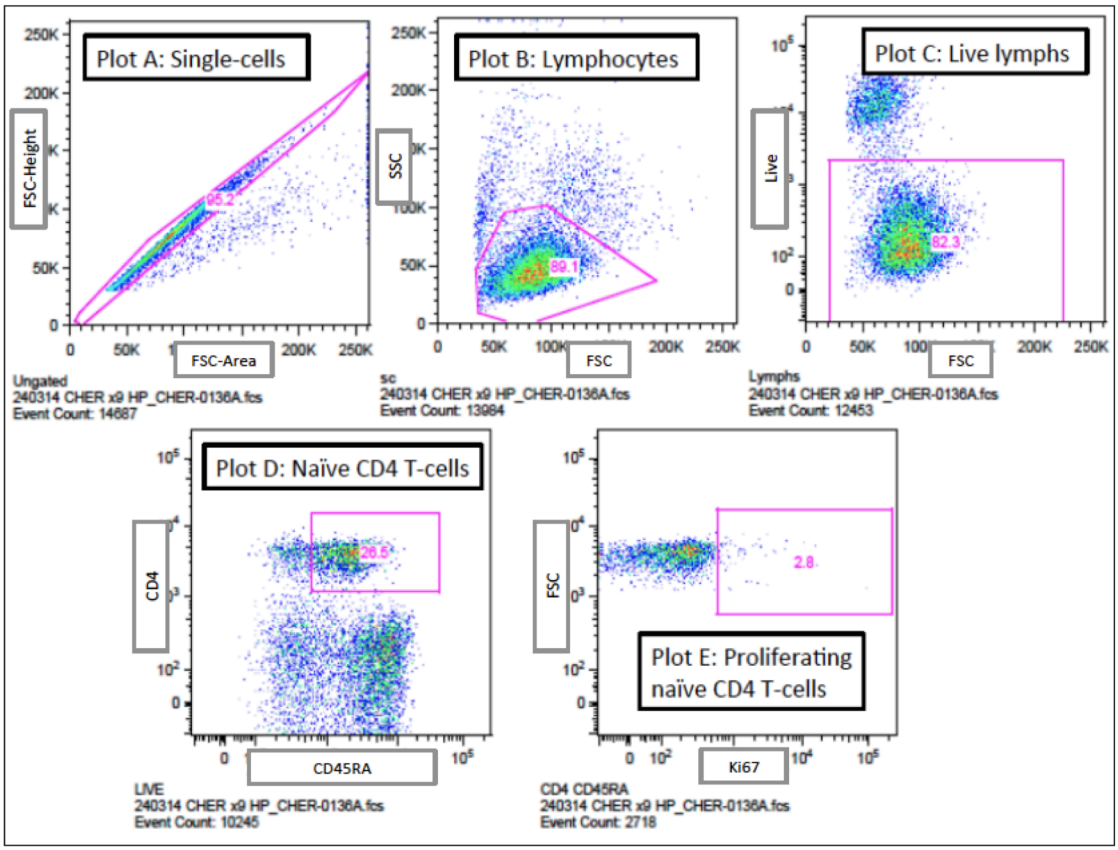
Antibody concentration was determined using titration experiments comparing the relative median fluorescence intensity i.e. the concentration that results in the highest signal of the positive population and lowest signal of the negative population [71]. Compensation was calculated by FACSDiva software, run weekly using PBMCs for the viability stain and compensation beads (BD CompBeads) for the fluorochromes.

Cell analysis was performed on a FACSCanto flow cytometer using FACSDiva software (BD Franklin Lakes, NJ) and data were analysed using FlowJo software (Tree Star, Ashland, OR). Single-lymphocyte events were gated by forward scatter area versus height, then side versus forward scatter for size and granularity, followed by gating on live cells using the viability marker. Depending on number of cells available, 20,000 to 50,000 gated lymphocytes were analysed. The gating strategies for panels A and B are described in detail in Appendices XIV and XV, and gate positions were determined using the fluorescence-minus-one approach [72]. An example of the gating strategy for measurement of proliferating naïve CD4 T-cells is given in Figure 2.5. The immunophenotypes measured in both panels are listed in Table 2.3.

Quality control measures applied to the gating strategies were as follows:

- Lymphosum: sum of CD3, CD19 and CD56 should amount to 90-110% to ensure the lymphocyte population has been appropriately captured) [73];
- CD3 from both panels should be identical ±3% (recognised as potential variation between repeated samples [74]);
- Weighted average calculations: subsets of CD4 and CD8 combined should equal the subset of the parent marker CD3 (±3%) i.e. CD3+HLA-DR+ cells should equal the sum of CD4+HLA-DR+ and CD8+HLA-DR+ per proportion of CD4/CD8; for example if CD4/CD8 ratio is 40:60 and CD4+HLA-DR+ T-cells are 5% and CD8+HLA-DR+ are 10%, CD3+HLA-DR+ T-cells will therefore comprise 8% of all CD3s ((0.4*5%)+(0.6*10%)=8%).

Figure 2.5: Gating strategy example for proliferating naïve CD4 T-cells.



Plot A gates on single-cells; plot B is a descendent of the single-cells, gating on lymphocytes by size and granularity; plot C gates on live lymphocytes not highlighted by the viability marker; plot D gates on CD4+ and CD45RA+ live lymphocytes; plot E gates on the proliferating naïve CD4 T-cells.

Preliminary work using fresh and frozen cells

To explore the effect of cryopreservation on immunophenotypes of interest, flowcytometry was performed on 43 pairs of fresh and frozen PBMCs. PBMCs were isolated by Lymphoprep gradient separation and divided into cells for immediate flowcytometry staining and analysis, and cells to be cryopreserved (as per section 2.4.1) for subsequent immunophenotyping. All samples were run on a BD FACSCalibur™, acquired using CellQuest™ Pro software and analysed using Flowjo™. The individuals comprised of 21 UK adults, 13 South African adults and 9 HIV-infected children from the UK.

Table 2.3: Immunophenotypes measured by panels A and B from frozen PBMCs.

Panel A	
Live %	Live lymphocytes
CD3+	T-cells
CD3+CD45RA+	Naïve T-cells
CD3+CD45RA+Ki67+	Proliferating naïve T-cells
CD3+CD45RA-	Memory T-cells
CD3+CD45RA-Ki67+	Proliferating memory T-cells
CD3+HLADR+	Activated T-cells
CD3+Ki67+	Proliferating T-cells
CD3+CD4+	T-helper cells (CD4)
CD3+CD4+CD45RA+	Naïve CD4 T-cells
CD3+CD4+CD45RA+Ki67+	Proliferating naïve CD4 T-cells
CD3+CD4+CD45RA-	Memory CD4 T-cells
CD3+CD4+CD45RA-Ki67+	Proliferating memory CD4 T-cells
CD3+CD4+HLADR+	Activated CD4 T-cells
CD3+CD4+Ki67+	Proliferating CD4 T-cells
CD3+CD8+	Cytotoxic T-cells (CD8)
CD3+CD8+CD45RA+	Naïve CD8 T-cells
CD3+CD8+CD45RA+Ki67+	Proliferating naïve CD8 T-cells
CD3+CD8+CD45RA-	Memory CD8 T-cells
CD3+CD8+CD45RA-Ki67+	Proliferating memory CD8 T-cells
CD3+CD8+HLADR+	Activated CD8 T-cells
CD3+CD8+Ki67+	Proliferating CD8 T-cells
Panel B	
Live %	Live lymphocytes
CD3+	T-cells
CD19+	B-cells
CD19+Ki67+	Proliferating B-cells
CD19+IgD+CD27-	Naïve unswitched B-cells
CD19+IgD+CD27-Ki67+	Proliferating naïve B-cells
CD3-CD56+	Natural killer cells
CD3-CD56+Ki67+	Proliferating natural killer cells

2.4.3 Whole blood assay for fresh blood immunophenotyping

Immunophenotypic analysis by whole blood assay (WBA) on fresh blood was performed to establish immunological reference ranges in South African children. This was done in the NHLS flowcytometry laboratory at Charlotte Maxeke Johannesburg Academic Hospital by the team lead by Denise Lawrie.

Blood samples were taken at the CWC between 9am and 1pm and 500µl were put into an EDTA tube, couriered at room temperature by air to Johannesburg and processed the following morning according to standard operating procedures. Directly labeled antibodies, including CD3 APC, CD3 FITC, CD16 PE, CD19 FITC, CD45 PerCP, CD45RO PE, CD45RA FITC, HLA-DR APC (Becton Dickinson Immunocytometry Systems (BDIS)), CD4 FITC, CD8 PE and CD56 PE (Beckman Coulter) were added in pre-titrated manufacturer optimised concentrations to tubes with 50µl of well-mixed whole blood. Stained samples were vortexed once and incubated for 30 minutes, after which the red blood cells were lysed using FACS Lysing Solution (BDIS). All samples were run on a Becton Dickinson FACSCalibur™, acquired and analysed using CellQuest™ Pro software.

Prior to analysis, basic daily flow cytometer set-up included assessment of Calibrite™ 3 and Calibrite™ APC beads (BDIS) to monitor laser, optics, fluidic alignment, linearity and instrument performance for the FACSCalibur™, according to the manufacturers' standards. Listmode data was stored for retrospective analysis. External CD4 Quality Assessment for CD4 testing was performed through the UK National External Quality Assessment Service (NEQAS) Immune Monitoring scheme and the NHLS CD4 African Regional External Quality Assessment Scheme [75]. Lymphocyte subsets were expressed as a proportion of total lymphocytes, which was determined using bright CD45 expression and side scatter. Specific lymphoid subsets assessed included: CD3+, CD3+CD4+, CD3+CD8+, CD3-CD56+, CD16+56+, CD3+HLADR+, CD3+CD4+HLADR+, CD3+CD8+HLADR+, CD3+CD4+45RA+, CD3+CD4+45RO+, CD3+CD8+45RA+, CD3+CD8+45RO+ and CD19+ lymphocytes. Absolute cell counts were obtained using a dual platform method and total lymphocyte counts on all samples were obtained on a Beckman Coulter LH750 haematology analyser. All laboratory work and data analysis performed were blinded.

2.4.4 Thymic Output

The mathematical formula used in this study to quantify thymic output combines measures of TRECs from purified naïve CD4 T-cells, absolute numbers of naïve CD4 T-cells and Ki67 data to calculate the rate of peripheral naïve T-cell proliferation [11]. The formula devised by Bains et al used TRECs to determine the relative contribution of thymic export and post-thymic expansion and Ki67 expression to estimate the contribution of peripheral division.

Naïve CD4 T-cells were identified by staining for CD4+CD45RA+ lymphocytes, and dividing naïve CD4 T-cells were determined using the proliferation marker Ki67 [76]. Ki67 is only expressed in proliferating cells in late stage G_I then is subsequently rapidly degraded [76] therefore it can be used to represent the population of dividing cells. The dynamic formula for thymic export in terms of total naïve cell numbers, naïve cell TREC content and Ki67 expression is given as:

Equation 1: Thymic Export (cells day^{-1}) = $$\theta(t) = \left(\tau(t) \frac{y(t)}{\Delta} + \frac{d\tau(t)}{dt} \right) \frac{N(t)}{c - \tau(t)}$$

Where y(t) is the fraction of naïve CD4 T-cells expressing Ki67, Δ is the duration of Ki67 expression, τ (tau) is the TREC content of the peripheral naïve CD4 T-cell population, c is a constant representing the average TREC content of thymocytes entering the peripheral naïve population (0.6) [77], and N(t) is the total size of the naïve CD4 T-cell pool. The mean division of naïve CD4 T-cells is estimated to be 12.4 hours (±0.97 hours), therefore Δ is made the constant value of 0.52 (i.e. 12.4/24) [78]. N(t) is estimated by multiplying the number of naïve CD4 T-cells per microlitre of blood by the total body volume of blood [0.97 x Log(body weight in kg)+4.93] [79], then dividing this figure by 0.02 as blood lymphocytes account for approximately 2% of the body's total lymphocyte population.

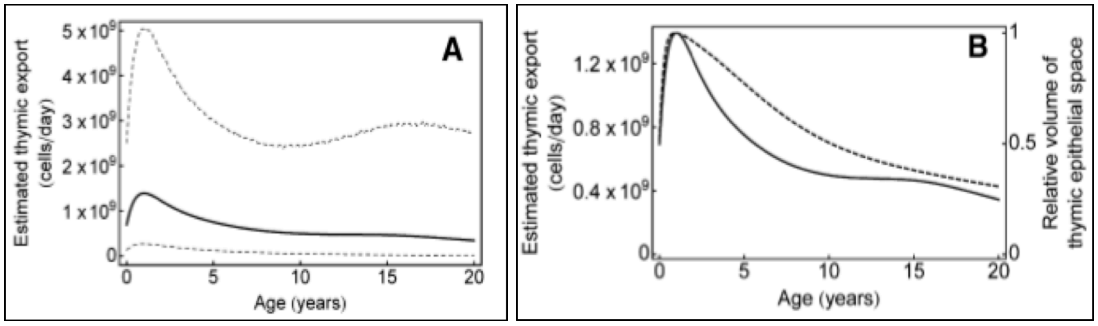
Equation 1 is a dynamic formula and relies upon more than one measurement in time. If thymic export is to be estimated at a single time-point the formula simplifies to Equation 2 as below:

Equation 2: $$\theta(t) = \frac{y \times N \times \tau}{\Delta(c - \tau)}$$

For example, a 3 year old child with a weight of 10kg will have a blood volume of 800,000µl based on the formula [0.97 x Log(body weight in kg)+4.93]. A full blood count revealed a lymphocyte count of 2.8 x 10^9/L and flowcytometry on PBMCs showed 28.7% naïve CD4, therefore it is estimated that there are 795 naïve CD4 T-cells per microlitre of blood and total of 3.2 x 10^{10} naïve CD4 T-cells in the body. This is calculated by blood volume multiplied by naïve Cd4 T-cells per microlitre of blood, then divided by 0.02 (since 2% of the body's lymphocytes are in the blood). Flowcytometry reveals a proliferation proportion of 1.4% of naive CD4 T-cells which are positive for Ki67. DNA PCR performed on the purified naïve CD4 T-cells showed 0.05 TRECs per naïve CD4 T-cell. This information put into equation 2 produces the explicit estimate of thymic output i.e. (0.014 x (3.2 x 10^{10}) x 0.05) / (0.52 x (0.6 – 0.05)) = 7.8 x 10^7 naïve CD4 T-cells exported from the thymus per day.

This model of thymic output was produced by Bains et al using paediatric data from two independent studies: Hazenburg et al [80] from The Netherlands (n=25, age range 0.3-16 years) and Douek et al [81] from the USA (n=12, age range 0-20 years). The model [66, 67] is comparable to estimates of thymic output from size and cellularity of paediatric thymuses from birth to 20 years [82-85] (Figure 2.6).

Figure 2.6: Mathematical modeling of thymic output by Bains et al [11].



A) Estimated rate of thymic export of CD4 T-cells (solid line=mean, dashed lines=2.5 and 97.5% quantiles). B) Estimated thymic output (solid line) and volume of thymic epithelial space (dashed line).

Naïve CD4 T-cell isolation

Purified naïve CD4 T-cells were obtained from the naïve T-cell isolation kit II (Miltenyi Biotec, Germany). Non-T helper cells and memory CD4 T-cells were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as a primary labeling reagent, and anti-biotin microbeads, as a secondary labeling reagent. The cocktail included biotin-conjugated monoclonal antibodies against CD8,

CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and CD235a (Glycophorin A). Both incubation times were increased from the manufacturer's instructions of 10 and 15 minutes to 30 minutes for both incubations to maximize purity. This was validated with a series of experiments and ensured that purity was consistently greater than 95%. The magnetically labeled cells were depleted as the solution was passed through MACS® LS columns in the magnetic field of a MACS separator, while the unlabeled naïve CD4 T-cells were eluted through the column and collected beneath. (Appendix X: Thymic Output SOP).

DNA extraction of naïve CD4 T-cells

DNA was extracted from purified naïve CD4 T-cells following isolation using the QIAGEN® QIAmp DNA extraction kit. After washing twice in PBS, cells were lysed with AL lysis buffer and digested with proteinkinase K at 56°C for 10 minutes. Ethanol was added and the sample was applied to a QIAmp mini spin column. The column was centrifuged at full speed, and filtrate reapplied to the column and spun again, washed twice with buffers AW1 and AW2 followed by buffer-free spin to remove buffer traces that might inhibit subsequent PCR. Extracted DNA was eluted from the mini spin column in AE buffer and stored at -20°C until PCR. Following the manufacturer's instructions yield of DNA was approximately 60% for lymphocytes.

TREC quantification

T-cell receptor excision circles (TRECs) were measured from DNA extracted from naïve CD4 T-cells by a real-time quantitative PCR assay using primers and fluorogenic probes specifically designed for the detection of human TRECs. The technique uses standard PCR reaction methods [86] and separate primer pairs for the amplification of TRECs and a housekeeping gene, the T-cell receptor alpha-constant gene (TRAC). The fluorogenic probe consists of an oligonucleotide labeled with a fluorescent reporter dye (either FAM or VIC) and a fluorescent quencher (TAMRA). By running standard control amounts of TRECs and TRACs in triplicate on the 96-well plate a standard curve was generated and used to establish the number of TRECs and TRACs in each sample. The standard curve was created in a logarithmic dilution series using known copies of plasmid containing signal joint TREC (donated by Dr Stuart Adams, Cameliar Botnar Laboratories, Great Ormond Street Hospital). To ensure the reliability of multiple PCR runs, the standard curve requires a slope of between -3.1 and -3.6, y-intercept 36 to 41, and R^2 >0.97 to ensure accuracy of the results. Up to 20% of the standard curve may be omitted

however sample values measured above the highest point or below the lowest point of the standard curve cannot be reliable. The cycle threshold was consistently set at 0.03 and the baseline set 3 cycles below the first amplification for analysis of all runs. There are two copies of the TRAC gene per cell, therefore it is possible to deduce the ratio of TRECs per cell ratio to use in the thymic output formula.

TRECs were quantified by real-time PCR using the Applied Biosystems 7900HT Fast Real-Time PCR System (TaqMan, Life technologies). For each PCR reaction the assay includes 0.5µl of each primer at a concentration of 45µM, 0.5µl probes at a concentration of 10µM, 6µl nuclease free water and 12.5µl TaqMan Universal PCR Master Mix (Table 2.4 for primers and probes sequences). PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each standard and sample was run in triplicate with average TREC and TRAC values used for data analysis, a non-template control was used in every run and a run-control was used weekly. The run control used purified naïve CD4 T-cells from an adult, therefore tested the high-end of the standard curve for TRACs and low-end of the standard curve for TRECs (mean 0.005 TRECs per cell, SD 0.003). Samples were re-run when less than the 3 reactions amplified or if the values differed by greater than 2 standard deviations. (Appendix XI: TREC and KREC Quantification SOP).

Table 2.4: Primers and probes sequences for the quantitative TREC PCR assay.

Primer / Probe:	**Sequence:**
TREC forward	5í-CAC ATC CCT TTC AAC CAT GCT-3í
TREC reverse	5í-TGC AGG TGC CTA TGC ATC A-3í
TREC probe	5í-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-TAMRA-3í
TRAC forward	5í-TGG CCT AAC CCT GAT CCT CTT-3í
TRAC reverse	5í-GGA TTT AGA GTC TCT CAG CTG GTA CAC-3í
TRAC probe	5í-FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCC-TAMRA-3í

2.4.5 Naïve B-cell output

The thymic output formula has been adapted to estimate naïve B-cell output combining kappa-deleting recombination excision circles (KRECs) from DNA extracted from PBMCs**,** quantification and proliferation of naïve B-cells. Naïve B-cells are depicted by unswitched non-memory B-cells (CD19+IgD+CD27-) and therefore any proliferation captured by the intracellular marker Ki67 on this subset will reflect homeostatic proliferation and not antigen-induced proliferation. Equation 2 below is the same as the thymic output formula yet here represents output of naïve B-cells per day where y(t) is the fraction of naïve B-cells expressing Ki67, T (tau) is the KREC content of the peripheral naïve B-cell population, and N(t) is the total size of the naïve B-cell pool. N(t) is estimated by multiplying the number of naïve B-cells per microlitre of blood by the total volume of blood [0.97 x Log(body weight in kg)+4.93] [79], then dividing this figure by 0.02 as blood lymphocytes account for approximately 2% of the body's total lymphocyte population.

Equation 2: $$\theta(t) = \frac{y \times N \times \tau}{\Delta(c - \tau)}$$

There are two assumptions the naïve B-cell output model makes based on limited published data available: average division of naïve B-cell (Δ = 0.52) per day [78] and average KREC content of naïve B-cells entering the peripheral circulation (c = 0.6) [77]. The replication history of the naïve mature B-cell subset in human peripheral blood has been reported to be an average of 2 cell divisions per day [87] although this is in adults. There are no published literature that describes mean KREC content of naïve B-cells entering the peripheral circulation, therefore the mean TREC content constant used in the thymic output formula (c = 0.6) has been adopted.

In addition to using the modified thymic output formula to estimate naïve B-cell output, the following measures could also feasibly be used: KREC per PBMC ratio, KRECs per mililitre of blood; or using flowcytometry data as KRECs per million B-cells (CD19+ lymphocytes) or per million naïve B-cells (CD19+IgD+CD27- lymphocytes).

DNA was extracted from an aliquot of cryopreserved PBMCs using the QIAGEN® QIAmp DNA extraction kit as described in the previous section for the DNA extraction from purified naïve CD4 T-cells. KRECs were measured from DNA extracted from

PBMCs by real-time PCR using custom-made primers and probes specifically designed for the detection of human KRECs and the housekeeping gene, TRAC (Table 2.5). The same methodology for the TREC-TRAC assay was used for the KREC-TRAC assay including the master mix concentrations, standard curve, equipment, PCR conditions and principles of analysis (Appendix XI).

Table 2.5: Primers and probes for the quantitative KREC PCR assay.

Primer/Probe:	Sequence:
KREC forward	5í-TCC CTT AGT GGC ATT ATT TGT ATC ACT-3í
KREC reverse	5í-AGG AGC CAG CTC TTA CCC TAG AGT-3í
KREC probe	5í-VIC-TCT GCA CGG GCA GCA GGT TGG-TAMRA-3í
TRAC forward	5í-TGG CCT AAC CCT GAT CCT CTT-3í
TRAC reverse	5í-GGA TTT AGA GTC TCT CAG CTG GTA CAC-3í
TRAC probe	5í-FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCC-TAMRA-3í

In an attempt to put the significance of this novel measurement of naïve B-cell output in a clinical context, the relationships between naïve B-cell output estimates and vaccination responses to PCV7 and HiB were explored within individual CHER participants from the parallel study CIPRA-4 (Section 2.2).

2.4.6 HIV-antibody assays

HIV antibody was measured on all available stored plasma samples at CHER trial week 84 (aged approximately 92 [IQR 90.6 – 93.4] weeks) from 2 of the 3 trial arms: ART-96W and ART-Def, thereby comparing early-limited ART with deferred ART started on clinical disease progression criteria (Centers for Disease Control and Prevention severe stage B/C disease) or CD4 <25%. Early ART was initiation of ART 5.8-12 weeks of age (median age 7 weeks [IQR 7-8] for ART-96W) compared to deferring ART until beyond 12 weeks of age (median 23 weeks [IQR 18-32] for ART-Def). At trial week 84, maternal antibody would no longer be detectable, all children in ART-96W were on ART and 89% of ART-Def had received at least 12 weeks ART.

HIV antibody was measured using three methods from plasma stored at –80°C at trial week 84. All specimens were thawed, heat-inactivated at 56°C for 30 minutes and centrifuged at 2500 rpm for 5 minutes to remove cell debris. Supernatant was aspirated and aliquotted for 3 assays:

(1) Automated serology: 4th generation microparticle enzyme immunoassay (EIA) HIV antigen/antibody combination (Abbott AXSYM system).

(2) Rapid antibody test: HIV-1/2 qualitative immunochromographic rapid antibody test (Alere Determine®) assessed by an independent, blinded clinician.

(3) Anti-gp120 ELISA: a sensitive in-house enzyme-linked immunosorbent assay (ELISA) to quantify anti-gp120 IgG and total IgG.

Automated HIV serology

Automated serology was performed at SUN NHLS laboratory using a 4th generation microparticle enzyme immunoassay HIV antigen/antibody combination (Abbott AXSYM system) using recombinant HIV antigens and HIV p24 monoclonal antibodies coated on microparticles to capture antibodies against HIV-1/2 and HIV p24 antigen respectively. Presence or absence of HIV-1/2 antibodies and/or p24 antigen in the sample was determined by comparing the rate of fluorescence to a cutoff rate from the AxSYM HIV Ag/Ab Combo Index Calibrator [88]. “Weakly reactive” results were re-classified as seropositive for the purpose of binary analysis, however the difference between re-classification as seropositive or seronegative was demonstrated by logistic regression.

Rapid HIV-antibody tests

Rapid HIV-antibody tests were performed by Alere Determine® HIV-1/2 [89], an in-vitro, qualitative immunochromographic assay to detect antibodies to HIV-1/2 in human serum, plasma or whole blood. The results were read by an independent, blinded clinician with experience reading rapid HIV-antibody tests.

Quantitative anti-gp120 antibody ELISA

An in-house enzyme-linked immunosorbent assay (ELISA) established at the Johannesburg National Institute for Communicable Diseases was used to quantify anti-gp120 HIV-antibody [90, 91] (Appendix XII: Quantitative anti-gp120 antibody ELISA SOP). Two ELISAs were run in parallel, one measured anti-gp120 IgG (HIV-specific antibody), and the second measured total IgG. Ninety-six well Maxisorp ELISA plates were coated overnight with either anti-human IgG coating buffer or gp120 coating buffer for the respective two ELISAs. The plates were washed with PBS and Tween20 solution, and then blocked with blocking agent (5% goat serum, 5% milk, 0.05% Tween20 and PBS) for 1 hour before adding the standard dilution series and samples. Human IgG in goat serum or gp120 clade C monoclonal antibodies in dilution buffer, respectively, are diluted by a 3-fold series dilution for a 16-point standard curve (100 to $7x10^{-6}$ ug/µl for IgG, 200 to $1.4x10^{-5}$ ug/µl for anti-gp120). Aliquots of CHER plasma diluted 8-fold or more were added to the plate in 4-fold dilution series over 8 wells per sample. Following an hour's incubation, the plates were washed, incubated with goat anti-human total immunoglobulin-biotin (detection antibody) for a further hour; washed and incubated with anti-biotin-peroxidase antibody (capture antibody) for a final hour; thereafter washed and Tetramethylbenzidine (TMB) substrate added to each well. After 5 minutes, the reaction was stopped by sulfuric acid (1 molar) and the absorbance at 450nm read by a VersaMAX ELISA Plate Reader.

Controls

Each ELISA plate included one HIV seropositive and one seronegative control. Plasma from 18 HIV-uninfected unexposed, age-matched healthy South African children were used to determine the specificity of the anti-gp120 ELISA. All HIV-uninfected controls were previously confirmed to be HIV negative by automated serology, corroborated by maternal health records.

2.4.7 HIV proviral DNA quantification

HIV-1 proviral DNA, referred to as HIV proviral DNA throughout this text, was measured by quantitative PCR from DNA extracted from 334 samples of cryopreserved PBMCs collected at 12-weekly time-points from 40-252 weeks during the CHER trial (Table 2.2). The HIV proviral DNA PCR assay measures long terminal repeats (LTR) and does not differentiate between integrated and episomal LTR. Integrated DNA is commonly used to estimate the size of the latent reservoir; whereas episomal DNA reflects on-going replication [92] and is believed to contribute little to persistence since integration is required for production of HIV proteins and subsequent infectious virus [93]. To minimize inclusion of episomal DNA and avoid bias, samples were used from all virally suppressed children on ART for a minimum of 24 weeks with two consecutive viral loads below 400 copies/mm^3 at least 3 months apart [92]. Therefore any episomal DNA measured should be negligible [92]. Using these criteria, all available specimens from 40 (ART-96W), 96 (ART-Def and ART-96W) and 248 (all arms) weeks of the trial were included. Fifteen children from ART-Def who fulfilled these criteria at 3 or more time-points (96, 156, 204, 248 and 252 weeks) were also included to describe patterns of proviral DNA decline on continuous ART.

Cryopreserved PBMCs were thawed to room temperature and DNA extracted using the QIAGEN® QIAmp DNA extraction kit, as described for DNA extraction of naïve CD4 T-cells (section 2.4.4, Appendix X).

Total HIV proviral DNA as copies of provirus were measured by real-time quantitative PCR assay using custom-made primers and fluorogenic probes (Table 2.6). The assay used standard PCR reaction methods [86], and separate primer pairs for the amplification of the region between LTR and gag, and pyruvate dehydrogenase (PDH) as the house-keeping gene. The standard curve was generated using a 6-point logarithmic scale of 8E5 cell-line from 1 to 10^5 copies per reaction. 8E5 is a cell line produced by American Type Culture Collection (ATCC), ideal for this purpose since there is exactly one copy of HIV provirus per cell [94]. To ensure reliability on multiple PCR runs the criteria for the standard curve include a slope of -3.1 to -3.6, y-intercept 36 to 41, and R^2 >0.97. Sample values measured above the highest point or below the lowest point of the standard curve may be unreliable. Both samples and standards were run in triplicate, and repeated if any of the standard curve criteria

were not fulfilled or if amplification was not in triplicate. Any undetectable measures of LTR were repeated for confirmation.

HIV proviral DNA was quantified by real-time PCR using the Applied Biosystems 7900HT Fast Real-Time PCR System (TaqMan, Life technologies). For each 25µl PCR reaction the assay includes 12.5µl of QIAgen Multiplex PCR Master Mix, 0.25µl of each PDH primer at a concentration of 10µM, 0.25µl of LTR primers at a concentration of 20µM, and 0.25µl of each PDH and LTR probe at a concentration of 10µM. To maximize the sensitivity of the assay 600ng of extracted DNA were added to each reaction well and the volume of extracted DNA made up to 11µl with nuclease free water. The maximum sensitivity of the assay was therefore 1 copy per 10^5 PBMCs, with results reported per 10^6 PBMCs in line with current publications.

PCR conditions were 95°C for 15 minutes, followed by 45 cycles of 94°C and 60°C for 1 minute each. A non-template control was used in every run, and each standard and sample were run in triplicate with average values used for data analysis. Samples were re-run when less than the 3 reactions amplified or values differed by greater than 2 standard deviations. There are 2 copies of PDH per cell, and although there are 2 LTR circles in each HIV gene only one is detected in this assay, hence the primer concentration is doubled for LTR. Therefore the explicit quantity of provirus per 10^5 PBMCs is the output value of LTR divided by PDH (Appendix XIII: HIV Proviral DNA quantification SOP).

Table 2.6: Primers and probes used for quantitative HIV proviral DNA PCR assay.

Primer / Probe	Sequence:
PDHTaq1 primer	5’ -TGA AAG TTA TAC AAA ATT GAG GTC ACT GTT -3’
PDHTaq2 primer	5’- TCC ACA GCC CTC GAC TAA CC -3’
PDHTaqPR probe	JOE-CCC CCA GAT ACA CTT AAG GGA TCA ACT CTT AAT TGT TAMRA
HIV1LTRTaq1 primer	5’- GCC TCA ATA AAG CTT GCC TTG A- 3’
HIV1LTRTaq2 primer	5’ -GGC GCC ACT GCT AGA GAT TTT- 3’
HIV1TaqPR probe	FAM -TGT GAC TCT GGT AAC TAG AGA TCC CTC AGA C TAMRA

Relationships were explored between HIV proviral DNA and clinical or immunological characteristics from the CHER trial, including 3-6 monthly viral loads, CMV DNA-status and quantification at baseline, serostatus and quantitative HIV-specific antibodies at trial week 84, and where available, immunophenotypes such as naïve

and memory proportions of CD4 and CD8 T-cells, activation and proliferation subsets.

Preliminary work on HIV-infected children in the UK

Preliminary work using the HIV proviral DNA assay was performed on 30 HIV-infected children and adolescents from a London clinic. To try to maximize the sensitivity of the assay, 22 samples were divided into aliquots of PBMCs, and PBMCs for MACs® isolation of memory CD4 T-cells. Four of these samples were further divided into memory CD4 T-cells and central memory CD4 T-cells. Memory CD4 T-cells were isolated by negative selection using magnetic bead separation, and central memory CD4 T-cells were further separated by positive selection using CD197 (CCR7)-PE and Anti-PE MicroBeads. DNA was subsequently extracted from these purified subsets and the aliquots of PBMCs.

Two samples of DNA extracted from PBMCs had undetectable provirus, although provirus was detected in the purified memory CD4 T-cells confirming the increased sensitivity of this approach. However, due to the division required the quantity of DNA per PCR reaction well was suboptimal (i.e. less than 600ng), thereby compromising the overall sensitivity of the assay, therefore subsequent analysis of CHER samples was performed on DNA extracted from PBMCs only. Excluding children who were virally unsuppressed, no relationship was identified between levels of HIV proviral DNA and last CD4%, proportion of life on ART or years before starting ART (n=19, p-values all >0.05).

Neurocognitive state and HIV proviral DNA

CHER participants at KIDCRU underwent Griffith's neurocognitive assessments at approximately 2 and 5 years of age including locomotor, personal/social, language, eye-hand coordination, and pattern reasoning performance tests. CNS macrophages, astrocytes and dendritic cells are recognised to harbour latent HIV, therefore the results of the Griffith's assessment have been analysed with HIV proviral DNA quantification from 96 and 248 weeks of the trial (n=29 and 65) to explore whether peripheral measures of HIV proviral DNA might reflect HIV-associated CNS disease and impact upon neurocognitive performance.

2.5 Statistical analysis

Sample size

At the time of designing this study there were no existing data on the variability of thymic output therefore estimation of sample size was inherently compromised. TREC data from a substudy of 33 HIV-infected children starting ART in the PENTA 5 trial [95] was instead used to calculate the number of samples required to give sufficient power for the comparisons in this study. The power to detect a given difference in Log10(TRECs) between two groups at a 5% significance level, assuming standard deviation (SD) of 0.5 for Log10(TRECs) has been calculated for the following sample sizes (Table 2.7). A sample size of 40 was chosen for the comparisons of thymic output in this study.

Table 2.7: TRECs variability, power and sample size calculation done with assistance from Professor Babiker and Trinh Duong at the MRC Clinical Trials Unit.

Difference in log (TRECs)	Number of children per group		
	30	40	50
0.3	0.61	0.73	0.82
0.4	0.85	0.93	0.97
0.5	0.96	0.99	0.998

Statistics for immunological reference ranges, international comparisons and fresh-frozen analysis

Exponential regression curves were produced with the assistance of Dr Martin Nieuwoudt. The cell counts or percentages of each lymphocyte subset were regressed against age for a continuous exponential curve using a three-parameter model: $Y(t) = B_0 + B_1 * (1 – \exp(B_2 * t))$. t is age in weeks and the Betas ($B_{0,1,2\ etc.}$) are the constants in the equation describing each lymphocyte subset. Best-fit (median) and 95% CI parameters were determined by minimizing the sum of the square of residuals using MS-Excel's Solver function with the Generalized Reduction Gradient nonlinear solver engine and a constraint precision of 0.0001. To account for the non-normal distribution of the residuals, upper and lower 95% confidence intervals (CIs) were fitted to the data independently. Comparisons were made between data from CWC and two international studies (Comans-Bitter et al from The Netherlands and Shearer et al from The USA) using the Chi-squared test, and for overall distributional differences between the median values of each cell group using the non-parametric Wilcoxon Rank Sum test. Subgroups of children with certain clinical characteristics

from the CWC were examined to determine whether their lymphocyte subsets differed from the rest of the CWC participants. This was achieved by comparing exponential regression fits and Wilcoxon Rank Sum tests for the subgroups with the remaining children from the cohort. For the reference intervals, median and 95% percentiles were calculated from both the haematological and immunological data sets stratified by age group.

Comparison of fresh WBA and cryopreserved PBMC was done by: Welch's t-test, visual comparison of fitted exponential curves, comparing the overall distribution of the groups using Wilcoxon Signed Rank tests, and by Bland Altman graphs with linear fits for each paired group. Mean percentage and direction of change from fresh to frozen was calculated by:

Equation 3: $$\left[\sum_{i}^{n}\left\{\frac{(fresh-frozen)i}{fresh\,i}\,x\,100\right\}\right]/n$$

The survival ratio was introduced to examine the direction and magnitude of change from the number of lymphocytes in a subset measured by fresh WBA to that quantified by the cryopreserved PBMC assay. This was calculated by the number of cells from a subset measured by fresh WBA subtracted from the number of cells from the same subset measured by cryopreserved PBMCs, then divided by the number of cells from the subset by fresh WBA i.e. survival ratio = (fresh WBA – frozen PBMCs) / fresh WBA. Variables that might influence the integrity of thawed subsets were explored using Pearson's rank correlation. Chi-squared tests were used to compare median and IQRs of the difference seen between WBA versus cryopreserved PBMCs in each cell subset.

Thymic output analysis

Clinical data from the CWC including gender, infant feeding practices, weight-for-age Z-score, vaccination status, recent illnesses, and relevant antenatal, neonatal and medical events were explored as factors that might influence thymic output. Thymic output in a log scale was compared between groups using the t-test and ANOVA in unadjusted analyses, and linear regression for adjusted analyses. ART-96W and HIV-uninfected children were compared at selected time points at equivalent ages. A non-linear curve was fitted to the data acquired from healthy controls, and individual

measures from all CHER samples were then directly compared to this curve. The non-linear curve was generated with assistance from Dr Joanna Lewis using b-splines, minimising squared distance of the data from the line or by fitting a quantile regression which is robust to uncharacteristic distributions. The same formula was used to fit the distribution of CD4 counts from the healthy South African children of the CWC from which age-adjusted Z-scores were calculated, as the difference from expected CD4 count for age (X). The formula for a Z-score = $(X - \mu) / \sigma$.

Naïve B-cell output analysis

As per the thymic output analysis, naïve B-cell output was explored using the clinical and immunological data from the CWC and CHER, and a non-linear curve was fitted to the data from healthy controls as per the b-splines approach described above. Naïve B-cell output has been compared between groups using t-test and ANOVA in unadjusted analyses.

HIV-antibody analysis

Welch's t-test was used to compare the 3 HIV-antibody assay results between ART-Def and ART-96W. The chi-square test was used to further compare the two arms. Spearman's rank correlation to describe relationship between antibody quantity and cumulative viral load or age of ART initiation. Cumulative viral exposure at trial week 84 was summarised by area under the time-versus-log(viral load) curve, from randomization to week 84. This measure was calculated in each child from the available viral loads using the trapezium rule [96]. Logistic regression was used to model the relationship between the probability of a positive automated serology result and the following variables: age at ART initiation, time on ART by trial week 84, CD4 count/percentage, cumulative viral load and clinical progression.

HIV proviral DNA analysis

Log-transformation was undertaken to provide normalized data where appropriate. Welch's t-test was used to compare quantitative results between ART-Def and ART-96W, and ANOVA to compare results between the 3 trial arms. Pearson's correlation and regression analyses were used to measure the association between proviral DNA and weeks of ART adjusted for weeks of continuous HIV RNA suppression, CD4 count and quantitative HIV-specific antibody (anti-gp120 IgG). Duration of HIV RNA suppression was estimated by the sum of weeks between suppressed viral loads measured at 12-24 weekly intervals throughout the trial. Predictive factors were

assessed using a case-control design i.e. “cases” were children with <100 copies of provirus/10^6 PBMCs compared to “controls” who had >100 copies of provirus/10^6 PBMCs; children with undetectable proviral DNA were then compared to those with any detectable proviral DNA.

All analyses were performed using the software R version 2.15.1 [97].

Chapter 3

Immunophenotypes in healthy South African children

3.1 Background

The importance of appreciating the evolving dynamics of the paediatric immune system is indisputable, particularly its relevance for understanding the pathogenesis and recovery of immunological disorders. A fundamental element of this understanding is the knowledge of what is “normal” in the healthy child within their natural environment. This chapter addresses the deficit of local, contextually appropriate immuno-haematological reference intervals for South African children by establishing these reference ranges from a population of healthy South African children seen at the Child Wellness Clinic. Potential differences in normal immunological profiles between children in low and high disease-burdened settings have been described. Finally, since a large majority of immunological research is performed on cryopreserved specimens, the effects of cryopreservation on normal immunological parameters have also been examined.

3.1.1 Local reference ranges

A detailed knowledge of the normal range of haematological and immunological parameters in healthy infants and children from a the population in which they reside is not only essential for appropriate clinical practice, but is also vital for the interpretation of the immunological responses of HIV-infected children to different ART-strategies. It is not uncommon for healthcare providers within the laboratory and daily clinical care in resource-limited settings to use reference ranges adopted from studies that have acquired data from healthy children in resource-rich countries [99-101]. However, it is plausible that the influence of both genetics and environment could influence haematological and immunological profiles that are considered to be normal in healthy children. Since treatment guidelines such as antiretroviral therapy (ART) initiation are based on immunological parameters, it is crucial that these reference ranges are appropriate to the population for whom they will be used. South Africa's paediatric haematology and immunology reference ranges currently used in national clinical practice have been obtained from studies in children from the United States and Europe [99, 102, 103] and therefore may not be entirely applicable for South African infants and children considering the ethnic, genetic and environmental differences that exist [104].

Differences in haematological cell populations between industrialised and resource-poor countries have been reported in several studies [105-110] including non-genetic neutropenia in people of African origin [111], and lower platelet counts [112, 113] when compared to reference ranges from the US and Europe [99]. There are also a few published studies that have explored differences in lymphocyte parameters between resource-rich and resource-limited countries. In one study, lower CD4 and higher CD8 T-cell subsets [110], reduced naïve T-cell proportions and increased activated CD4 and CD8 T-cells were reported in Ethiopian compared to Dutch adults [114]. In another study, lower B-cell and higher NK-cells were demonstrated in Tanzanian, Ethiopian and Ugandan adults compared to German adults [115]. There is less data from African children. CD4 percentages have been reported to be lower in children from Cameroon [116], Kenyan [117] and Uganda [118] compared to reference ranges from resource-rich countries. This is supported by a Malawian study that showed 35% of healthy children under 1 year and 18% of children aged 1-3 years had CD4 percentages below World Health Organisation (WHO) thresholds and 13% of children under 18 months old had a CD4:CD8 ratio of <1.0 [119].

While local reference ranges in Africa are increasingly being established [105, 116, 117, 120, 121], the range of immunological parameters is often limited and statistical comparison across populations have not been explored in detail. Although it is recognised that lymphocyte populations change with age, the rate of change has also not been explored between populations internationally. The data produced from healthy South African children who participated in the Child Wellness Clinic has been published [98] and will be used to establish comprehensive local haematological and immunological reference ranges for all South African children including activation markers, naïve and memory subsets. In addition, these data have been used to explore the immunophenotypes and dynamics of children from two distinct environments, South Africa and three industrialised countries, Germany [122], The Netherlands [123] and The USA [103].

3.1.2 Research using cryopreserved peripheral blood mononuclear cells

Clear information regarding the effect of cryopreservation and comparing different approaches to immunophenotyping is invaluable for the interpretation of phenotypic studies performed on cryopreserved PBMCs. This is especially important, as the use of cryopreserved cells is the most practical option for laboratory research, particularly so in large clinical trials as it minimises operator dependent inter-assay variability and optimises available resources. The difficulties and controversies of laboratory research using cryopreserved PBMCs are frequently cited in published literature [124-127], especially in functional studies and examining T-regulatory cells. There are less conflicting views regarding phenotypic research using cryopreserved PBMCs [128-130], however study numbers are small and usually in adults.

The immunological data acquired from fresh whole blood analysis from blood samples from healthy South African children in this study have been compared with the same immunophenotypes measured from PBMCs cryopreserved from the same aliquot of blood. This analysis intends to determine whether preferential cell preservation or death occurs within lymphocyte subsets. The analysis aims to describe how common lymphocyte subsets change with cryopreservation and other differences in methodologies, whether some subsets change more than others, whether changes are constant or variable, and what factors may determine the integrity of thawed subsets i.e. proportion of live thawed cells, gender, age, recent illness and intercurrent infections such as HIV.

3.2 Results

3.2.1 Child Wellness Clinic population characteristics

The structure and organisation of the Child Wellness Clinic (CWC) is described in detail in Chapter 2, section 2.1. The distributions of maternal and infant age are illustrated in Figure 3.1, and the demographics and participant characteristics presented in Table 3.1. The child's weight, length and head-circumference were measured and weight-for-age Z-score recorded from the child's Road-to-Health card (hand-held medical records). These growth charts are derived from The National Centre for Health Statistics standards from US surveillance data [131]. The weight-for-age Z-score was approximately normally distributed around -1 to 0 of the reference population (Figure 3.2).

Figure 3.1: CWC distribution of: A) Maternal age at child's birth; B) Age of children.

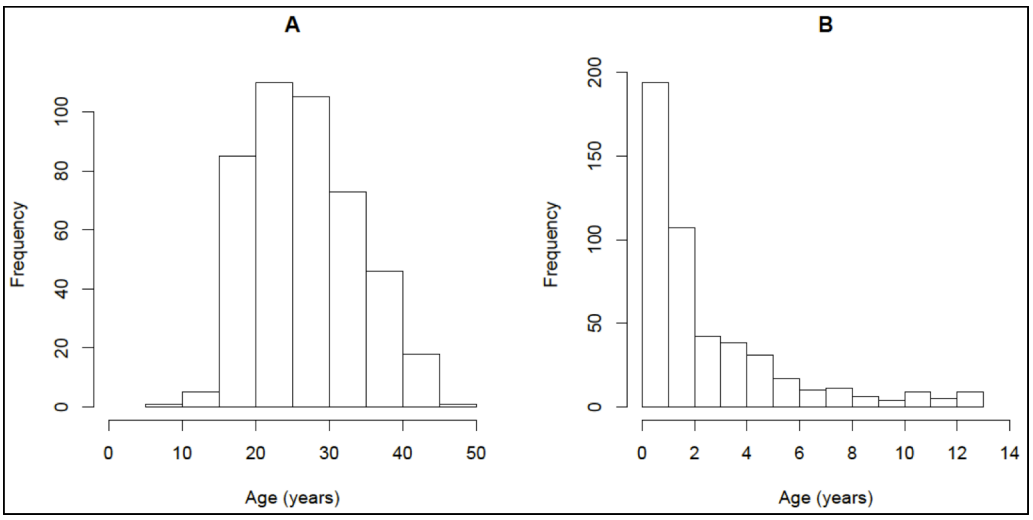
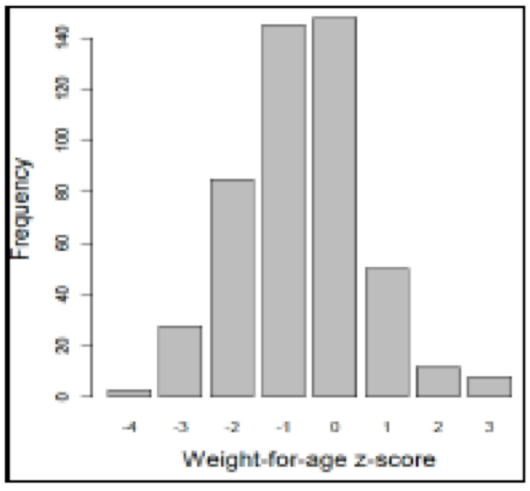


Figure 3.2: Distribution of weight-for-age Z-score of CWC participants.



Z-score was taken from the weight plotted on the child's growth chart on their "Road-to-Health" hand-held medical records. A Z-score of 0 is equal to the mean, and scores above or below reflect the number of standard deviations (SD) from the mean of the reference population.

Table 3.1: Characteristics of Child Wellness Clinic participants (n=471).

	n (%) or median [IQR]
Decimal age at CWC	1.5 [0.5 – 3.4]
Maternal age at child's birth	25.6 [20.8 – 31.6]
Ethnicity	Black: 105 (22.3) Mixed ethnic ancestry: 356 (77.7)
Gender	Female: 243 (51.6) Male: 228 (48.4)
Antenatal event (No. & percentage of all CWC recruits)	46 (9.7) *Maternal HIV-infection 19 (4) – of which 3/18 did not receive PMTCT; Syphilis 10 (2.1); TB 4 (0.8); Mental illness 3 (0.6); Alcohol/drug abuse 4 (0.8); Hepatitis B 2 (0.4); UTI 2 (0.4); Diabetes 1 (0.2); STD 1 (0.2); Growth restriction 1 (0.2)*
Neonatal events (Events which required hospital admission or neonatal medication*)	52 (11) *Prematurity 28 (5.9); ART 16 (3.4); Suspected infection 5 (1.1); Jaundice 5 (1.1); LBW 3 (0.6); HIE 3 (0.6); Feeding difficulties 2 (0.4); Meningitis 1 (0.2); Pneumonia 1 (0.2); Eye infection 1 (0.2)*
Medical history (Past admission, chronic illness or poor growth*)	55 (11.7) *Pneumonia 14 (3.0); Gastroenteritis 10 (2.1); TB 10 (2.1); Faltering growth 7 (1.5); Seizures 4 (0.8); Asthma 2 (0.4); Croup 2 (0.4); PUO 2 (0.4); Pyelonephritis 2 (0.4); Other 6 (1.3): Bacteraemia, Herpes simplex, Meningitis, PUJ obstruction, Reactive arthropathy, Trauma*
Recent illness (>1wk ago but within past month*)	64 (13.6) *LRTI 24 (5.1); Gastroenteritis 15 (3.2); URTI 13 (2.7); Skin infection 8 (1.7); Otitis media 2 (0.4); PUO 2 (0.4); Measles 1 (0.2)*
No family/social support	8 (1.7)
Reported alcoholism, drug abuse or violence at home	11 (2.3)
Feeding in first 6 months	Exclusive breast feeding: 287 (60.9) Formula feeding only: 58 (12.2) Mixed breast and formula: 126 (26.8)
Growth status	Growing well: 438 (93) Growing well but history of poor growth: 8 (1.7) Plateau growth: 24 (5.1) Severe faltering growth: 1 (0.2)
Immunisation status	Fully immunized (records confirm): 400 (84.9) Fully immunized (parental verbal report): 4 (0.8) Immunisation not up-to-date: 67 (14.3)

*Some children had >1 event listed. NB. The one child with severe faltering growth was excluded from subsequent analysis.

464/471 (98.5%) infants had a rapid HIV-antibody test performed and all were negative. The remaining 7 infants were less than 18 months, HIV-exposed and had a record of a negative HIV DNA PCR in their clinic notes; therefore the HIV-antibody test was not performed. 14/19 HIV-exposed children had a negative PCR documented in the clinic files, the remaining 5 children had no record of PCR results however, they were all over the age of 2 years and rapid HIV-antibody test performed were all negative.

3.2.2 Local population reference ranges

Methods sections 2.4.1-3 describe specimen processing and immunology panels. The fresh whole blood assay that measured a detailed immune profile from 381 CWC recruits done by the NHLS at Charlotte Maxeke Johannesburg Academic Hospital, and the full blood counts performed by a BARC Global Central Laboratory for clinical trials, have been used to create immunology and haematology reference ranges for paediatric clinical practice in South Africa since the ranges currently used are derived from US and European studies. These reference ranges have adopted the age categories of Shearer et al [103] i.e. 0-3 months, 3-6 months etc.

Tables 3.2, 3.3 and 3.4 detail the reference ranges calculated from CWC data. Figure 3.3 demonstrates an alternative way to present this data using exponential regression graphs. The steep, exponential change of lymphocyte subsets with age, especially in infancy, is well-recognised and presentation of the healthy child data-set using exponential regression allows reference intervals to be read directly from the graph or be generated for the specific age of the child examined using appropriate laboratory software. Exponential fits were possible for all lymphocyte subsets examined in Panels A and B, as listed in Table 2.3. Absolute cell count curves for all lymphocyte subsets descended with age towards an asymptote, a straight line that continually approaches a given curve but does not meet it at any finite distance. Those for cell percentages either increased or decreased, but also tended to an asymptote. Further exponential graphs for these lymphocyte subsets are presented in section 3.2.3.

Table 3.2: Reference intervals for components of the full blood count from the CWC (n=381). Values are reported as medians with 95% confidence intervals (CI) from each age group measured.

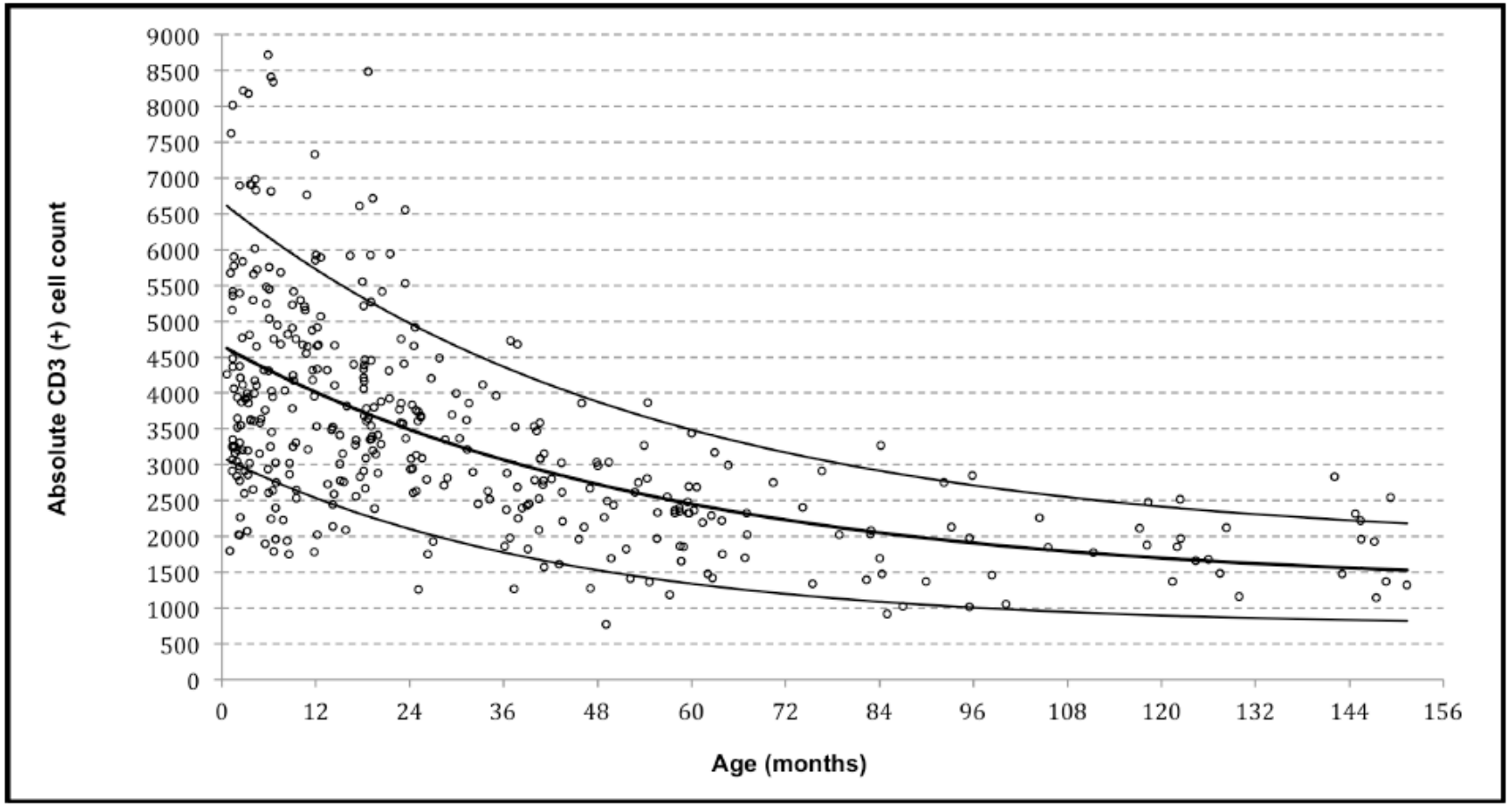
TABLE 3.2 Cell group (Median with 95% CI)	Age ranges 0-3 months (n=45)	3-6 months (n=40)	6-12 months (n=54)	1-2 years (n=79)	2-6 years (n=118)	6-12 years (n=37)	12+ years (n=8)
Red blood cells	3.5 (2.7-4.4)	4.2 (3.1-4.9)	4.4 (3.9-5.2)	4.4 (3.9-5.1)	4.3 (3.9-4.8)	4.2 (3.8-4.7)	4.6 (4.0-5.2)
Haemoglobin	10.6 (8.7-14.7)	11.4 (8.5-12.7)	10.8 (9.5-13.1)	10.8 (8.7-12.5)	11.2 (9.4-12.5)	11.8 (10.0-13.1)	12.8 (11.1-13.3)
Haematocrit	0.31 (0.25-0.41)	0.34 (0.25-0.38)	0.32 (0.28-0.39)	0.32 (0.27-0.37)	0.34 (0.29-0.38)	0.35 (0.31-0.38)	0.38 (0.32-4.00)
Mean corpuscular volume	92.2 (82.2-99.4)	79.2 (68.7-88.6)	73.5 (66.8-81.2)	72.5 (64.5-82.2)	78.9 (64.5-86.4)	83.2 (74.3-87.7)	81.1 (75.4-86.8)
Red cell distribution width	14.7 (12.8-18.2)	13.5 (11.7-16.8)	15.9 (13.4-21.3)	16.3 (14.1-20.8)	14.8 (12.8-19.4)	13.9 (12.7-15.9)	13.9 (13.3-16.3)
Platelets	420 (243-581)	390 (228-687)	367 (253-610)	349 (192-551)	326 (197-471)	319 (206-454)	280 (180-371)
White blood cells	9.6 (5.7-14.9)	10.4 (6.9-19.7)	12.0 (5.3-20.0)	10.7 (6.9-18.5)	8.7 (5.8-13.6)	7.6 (4.0-10.0)	8.6 (4.9-13.0)
% Granulocytes	24.1 (15.6-39.0)	29.6 (11.6-48.8)	31.7 (13.0-52.3)	31.5 (20.0-51.1)	42.0 (25.0-61.2)	48.2 (28.4-65.3)	54.4 (45.5-57.2)
Granulocytes (x10^9/L)	2.36 (1.26-5.20)	2.88 (1.22-8.11)	3.99 (1.13-8.08)	3.55 (1.79-7.87)	3.63 (1.59-6.64)	3.69 (0.85-6.33)	4.19 (2.60-7.30)
% Lymphocytes	60.0 (49.0-72.8)	58.8 (42.3-77.5)	58.0 (36.8-73.9)	57.0 (37.769.0)	43.4 (26.1-62.1)	37.5 (21.5-60.9)	35.1 (27.7-45.8)
Lymphocytes (x10^9/L)	5.56 (3.23-11.07)	6.12 (3.79-12.43)	6.20 (3.10-11.45)	5.75 (3.58-10.58)	3.81 (1.93-6.11)	2.71 (1.50-3.89)	2.85 (1.78-3.95)
% Monocytes	11.0 (5.0-18.3)	7.9 (3.2-16.6)	8.0 (2.8-13.4)	7.0 (3.0-12.0)	6.0 (2.5-11.0)	5.3 (1.6-11.2)	6.1 (4.2-8.0)
Monocytes (x10^9/L)	1.03 (0.52-2.40)	0.97 (0.32-2.37)	0.89 (0.30-1.93)	0.73 (0.29-1.61)	0.53 (0.20-1.06)	0.38 (0.12-0.97)	0.46 (0.22-0.75)
% Eosinophils	3.0 (1.0-6.3)	3.0 (0-10.0)	3.1 (1.0-8.0)	3.1 (0-10.0)	5.9 (1.5-18.1)	4.8 (0.9-12.9)	6.8 (1.1-16.0)
Eosinophils (x10^9/L)	0.25 (0.07-0.89)	0.33 (0-1.24)	0.29 (0.05-1.34)	0.32 (0-1.37)	0.51 (0.11-1.20)	0.27 (0.10-1.16)	0.50 (0.06-1.05)
% Basophils	0.2 (0-1.1)	0.2 (0.1.1)	0.3 (0-1.3)	0.2 (0-1.0)	0.3 (0-1.0)	0.3 (0-0.8)	0.3 (0-0.4)
Basophils (x10^9/L)	0.02 (0-0.11)	0.03 (0-0.12)	0.03 (0-0.17)	0.02 (0-010)	0.03 (0-0.08)	0.02 (0-0.04)	0.03 (0-0.10)

Table 3.3 and 3.4: Reference intervals for immunophenotypes from the CWC (n=381). Values are reported as medians with 95% confidence intervals (CI) from each age group measured. Table 3.3 details lymphocyte subsets by absolute cell count, in 10^9 cells per litre of blood. Table 3.4 details lymphocyte subsets in percentage of their parent subset however in cases of CD3+, CD3+CD4+ and CD3+CD8+ the parent subset is total lymphocytes.

TABLE 3.3: Cell group (Cells/μl with 95% CI)	Age ranges 0-3 months (N=45)	3-6 months (N=40)	6-12 months (N=54)	1-2 years (N=79)	2-6 years (N=118)	6-12 years (N=37)	12+ years (N=8)
CD3+	4548 (3003 – 6503)	4411 (2882-6305)	4182 (2682-5973)	3760 (2325-5364)	2894 (1658-4119)	1867 (990-2655)	1546 (828-2204)
CD4+	3060 (1962-4269)	2927 (1847-4111)	2711 (1666-3854)	2339 (1369-3397)	1697 (916-2553)	1144 (605-1729)	1039 (564-1538)
CD8+	1460 (722-2424)	1433 (711-2370)	1386 (691-2278)	1296 (650-2102)	1075 (537-1695)	712 (300-1090)	535 (144-831)
Ratio CD4+/CD8+	2.453 (1.223-3.756)	2.394 (1.201-3.667)	2.298 (1.167-3.518)	2.131 (1.111-3.250)	1.835 (1.028-2.729)	1.568 (0.973-2.169)	1.513 (0.967-2.018)
CD19+	1334 (498-3024)	1293 (492-2943)	1222 (482-2805)	1088 (460-2545)	782 (399-1972)	343 (255-1196)	163 (149-904)
CD3+HLA-DR+	420 (102-892)	411 (100-867)	396 (95-824)	365 (87-740)	288 (68-541)	151 (41-228)	77 (30-83)
CD4+HLA-DR+	139 (62-230)	136 (61-224)	131 (59-215)	121 (56-196)	96 (47-154)	58 (29-93)	41 (17-68)
CD8+HLA-DR+	239 (49-608)	236 (49-593)	229 (48—568)	215 (46-518)	176 (41-391)	86 (29-163)	20 (19-38)
CD4+CD45+RA+	2256 (1316-3258)	2151 (1236-3137)	1979 (1107-2936)	1675 (883-2573)	1124 (495-1864)	603 (158-1099)	488 (93-892)
CD4+CD45+RO+	723 (402-1109)	718 (400-1100)	709 (395-1084)	690 (386-1050)	636 (360-952)	509 (298-724)	416 (253-556)
Ratio CD4+naïve/memory	3.401 (1.900-5.146)	3.283 (1.813-5.003)	3.088 (1.669-4.763)	2.731 (1.412-4.312)	2.019 (0.926-3.341)	1.219 (0.429-2.078)	0.988 (0.305-1.627)
CD8+CD45+RA+	1094 (543-1795)	1075 (535-1759)	1042 (521-1698)	978 (492-1578)	816 (410-1294)	532 (224-843)	382 (91-633)
CD8+CD45+RO+	365 (114-737)	357 (108-719)	344 (99-687)	318 (83-629)	260 (54-500)	179 (26-326)	147 (19-260)
Ratio CD8+ naïve/memory	4.880 (1.355-10.561)	4.842 (1.360-10.303)	4.773 (1.369-9.876)	4.632 (1.389-9.101)	4.230 (1.444-7.584)	3.321 (1.573-5.935)	2.674 (1.667-5.482)
CD16+CD56+	645 (166-1292)	614 (160-1225)	566 (148-1118)	485 (127-941)	358 (83-670)	265 (27-479)	251 (8-453)
CD3-CD56+	643 (158-1385)	611 (127-1300)	562 (124-1169)	480 (124-954)	353 (124-638)	265 (124-432)	253 (124-408)

TABLE 3.4: Cell group (% with 95% CI)	Age ranges 0-3 months (N=45)	3-6 months (N=40)	6-12 months (N=54)	1-2 years (N=79)	2-6 years (N=118)	6-12 years (N=37)	12+ years (N=8)
CD3+	66.6 (56.0-76.3)	66.7 (56.4-76.3)	67.0 (57.0-76.4)	67.4 (58.0-76.4)	68.1 (58.6-76.5)	68.8 (58.9-76.7)	68.9 (58.9-76.8)
CD3+CD4+	42.1 (31.7-57.4)	42.0 (31.8-55.9)	42.0 (32.1-54.1)	41.9 (32.1-52.4)	41.6 (32.7-51.6)	41.0 (33.3-51.5)	41.0 (33.5-51.5)
CD3+CD8+	23.3 (12.8-30.4)	23.3 (13.3-30.7)	23.3 (14.1-31.1)	23.3 (15.4-31.6)	23.3 (17.0-32.0)	23.3 (17.9-32.1)	23.3 (18.0-32.1)
Ratio: CD4+ / CD8+	3.0 (1.1-4.3)	2.4 (1.1-4.0)	2.0 (1.1-3.5)	1.9 (1.1-2.9)	1.8 (1.1-2.6)	1.8 (1.1-2.5)	1.8 (1.1-2.5)
CD19+HLADR+	20.0 (11.9-29.3)	19.9 (11.9-29.0)	19.5 (11.8-28.5)	18.9 (11.6-27.3)	17.0 (10.9-24.3)	13.1 (8.4-17.8)	10.5 (5.6-13.5)
CD3+HLADR+	7.9 (2.6-15.5)	7.9 (2.6-15.3)	7.8 (2.5-15.1)	7.6 (2.5-14.6)	7.0 (2.5-13.3)	5.8 (2.3-10.2)	4.9 (2.2-8.1)
CD3+CD4+HLADR+	3.0 (1.2-4.9)	3.0 (1.3-5.0)	3.0 (1.3-5.0)	3.0 (1.4-5.0)	2.9 (1.5-4.8)	2.9 (1.6-4.6)	2.9 (1.6-4.5)
CD3+CD8+HLADR+	5.3 (0.2-12.0)	5.2 (0.6-11.9)	5.1 (0.6-11.6)	4.8 (0.6-11.0)	4.1 (0.6-9.3)	2.9 (0.6-6.0)	2.2 (0.6-3.7)
CD3+CD4+CD45RA+ (naïve CD4)	73.1 (61.9-83.5)	72.6 (61.3-83.1)	71.8 (60.3-82.4)	70.1 (58.1-80.8)	65.0 (51.8-76.4)	52.5 (36.5-65.8)	42.7 (24.7-57.9)
CD3+CD4+CD45RO+ (memory CD4)	25.7 (15.4-37.4)	26.4 (16.1-38.1)	27.7 (17.2-39.3)	30.2 (19.3-41.8)	36.5 (24.8-48.6)	47.5 (34.4-62.8)	53. (39.4-72.2)
Ratio: naive CD4 / memory CD4	3.4 (1.7-5.2)	3.3 (1.6-5.0)	3.1 (1.6-4.6)	2.7 (1.4-4.0)	2.0 (1.1-2.9)	1.2 (0.6-2.0)	1.0 (0.3-1.8)
CD3+CD8+CD45RA+ (naïve CD8)	77.1 (63.1-89.5)	77.1 (63.0-89.4)	77.0 (63.0-89.3)	76.6 (62.8-89.0)	75.8 (62.4-88.1)	74.1 (61.6-85.9)	72.9 (61.0-84.4)
CD3+CD8+CD45RO+ (memory CD8)	22.8 (10.5-36.9)	22.9 (10.6-37.0)	23.1 (10.7-37.0)	23.3 (11.1-37.2)	24.2 (12.0-37.6)	26.0 (14.1-38.5)	27.2 (15.7-39.1)
Ratio: naive CD8 / memory CD8	4.8 (1.4-9.3)	4.8 (1.4-9.3)	4.7 (1.4-9.2)	4.6 (1.4-8.9)	4.3 (1.4-8.2)	3.6 (1.5-6.7)	3.1 (1.6-5.5)
CD3-CD16+CD56+	8.3 (3.2-14.7)	8.3 (3.2-14.7)	8.4 (3.3-14.7)	8.5 (3.3-14.7)	8.9 (3.6-14.9)	9.7 (4.2-15.2)	10.3 (4.6-15.4)
CD3-CD56+	8.3 (3.2-14.4)	8.3 (3.2-14.4)	8.4 (3.3-14.5)	8.5 (3.3-14.6)	8.9 (3.6-15.0)	9.7 (4.2-15.9)	10.3 (4.6-16.5)

Figure 3.3: Example of exponential regression graphs for CD3+ T-lymphocytes.



Central line is the best-fit of the data and within the two surrounding two lines is 95% of the data. Y-axis is the absolute cell count is in T-cells per microlitre of blood.

3.2.3 International comparison of lymphocyte subsets

381 of the 471 children recruited from the CWC were included in this international comparison study and population characteristics were similar to those described in Table 3.1 for all 471 children who were seen and had phlebotomy performed.

Three independent studies have been used to compare the lymphocyte subsets from the CWC population of healthy South African children with those from the US or Europe: Shearer et al (North America [103]), Comans-Bitter et al (The Netherlands [123]) and Huenecke et al (Germany [122]). Shearer et al and Comans-Bitter et al used age-categorisation albeit different choices of age groups i.e. 0-3 months or 0- 2 months, whereas Huenecke et al presented their data using exponential regression curves. The CWC lymphocyte populations have therefore been compared according to the presentation of data in each publication. These US and European studies were selected for comparison as the first two are currently used for reference ranges in South Africa, and the latter enabled comparison of the populations using exponential regression techniques. All three studies used a whole-blood lysis assay to measure the lymphocyte subsets of interest. The immunological parameters available for comparison are listed in Table 3.5.

Clinical conditions included as normal for this population

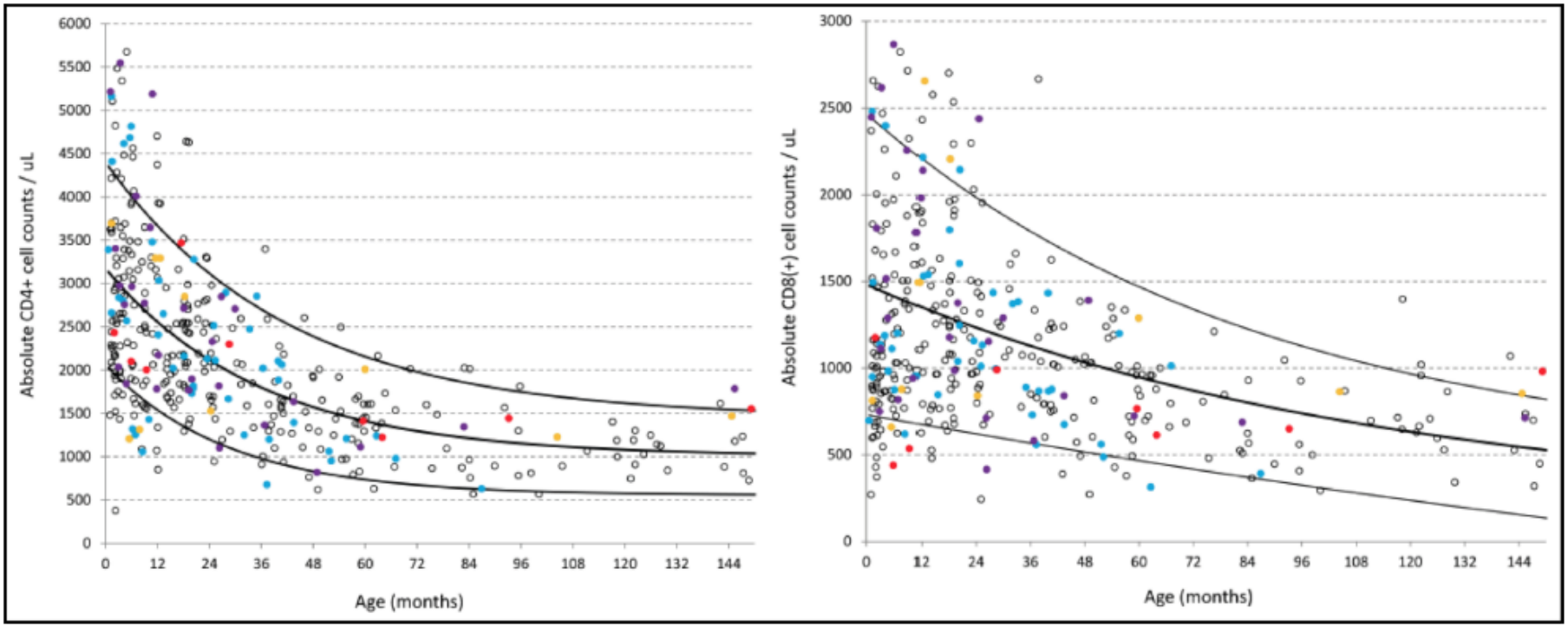
Figure 3.4 illustrates the distribution of CD4 and CD8 (cells/μl) from the CWC recruits including subgroups of clinical conditions common in this population, that might be presumed to affect the child's developing immune system and thereby influence the spread of data and reference ranges derived, although no evidence currently exists to confirm that this is the case. These conditions include a) past history of a serious childhood illness e.g. TB, meningitis (n=11); b) acute recent illness within the past month but more than a week ago e.g. upper respiratory tract infections, gastroenteritis (n=69); c) maternal conditions during pregnancy e.g. TB, HIV, syphilis (n=28); and d) prematurity <32 weeks (n=13). The exact exponential fit of the regression line for each lymphocyte subset examined (as per CWC column of Table 3.5) did not alter when each of the four clinical subsets were in turn removed from the analysis therefore justifying the inclusion of these children

Table 3.5: Immunophenotypes compared between international studies. Corresponding immunophenotypes were compared between the South African cohort (CWC) and three published studies: Comans-Bitter et al [123], Shearer et al [103] and Huenecke et al [122]. Both cell count/µl and percentage was available for each subset.

Subset	CWC	Comans-BItter et al	Shearer et al	Huenecke et al
No. children	381	358	851	80
Age range	2 wks – 13 yrs	1 wk – 16 yrs	Birth – 18 yrs	2 mths – 18 yrs
Country	South Africa	Netherlands	USA	Germany
Total lymphocytes	SSC/CD45 and FSC/SSC	FSC/SSC	FSC/SSC	FSC/SSC
T-lymphocytes	CD3+	CD3+	CD3+	CD3+
T-helper	CD3+CD4+	CD3+CD4+	CD4+	CD3+CD4+
Cytotoxic	CD3+CD8+	CD3+CD8+	CD8+	CD3+CD8+
CD4/CD8 ratio	CD3+CD4+ / CD3+CD8+	CD3+CD4+ / CD3+CD8+	NA	CD3+CD4+ / CD3+CD8+
Naïve CD4+	CD4+CD45RA+	NA	CD4+CD45RA+	CD3+CD4+CD45RA+
Memory CD4+	CD4+CD45RO+	NA	CD4+CD45RO+	CD3+CD4+CD45RO+
Naïve/Memory ratio of CD4+	CD4+CD45RA+ / CD4+CD45RO+	NA	NA	CD3+CD4+CD45RA+ / CD3+ CD4+CD45RO+
Naïve CD8+ T-cells	CD8+CD45RA+	NA	CD8+CD45RA+	CD3+CD8+ CD45RA+CD28+
Memory CD8+	CD8+CD45RO+	NA	CD3+CD4- CD45RO+	CD3+CD8+CD45RO+
Naïve/Memory ratio of CD8+ T-cells	CD8+CD45RA+ / CD8+CD45RO+	NA	NA	CD3+CD8+CD45RA+ CD28+ / CD3+CD8+CD45RO+
Activated T-cell	CD3+HLADR+	CD3+HLADR+	NA	NA
Activated CD4+	CD3+CD4+HLADR+	NA	CD4+HLADR+	CD3+CD4+HLADR+
Activated CD8+	CD3+CD8+HLADR+	NA	CD8+HLADR+	CD3+CD8+HLADR+
CD19+ B-cells	CD19+HLA-DR+	CD19+	CD19+	CD19+
Natural Killer (NK) cells	1) CD3- CD16+CD56+ 2) CD3-CD56+	1) NA 2) NA	1) CD16+CD56+ 2) NA	1) NA 2) CD3-CD56+

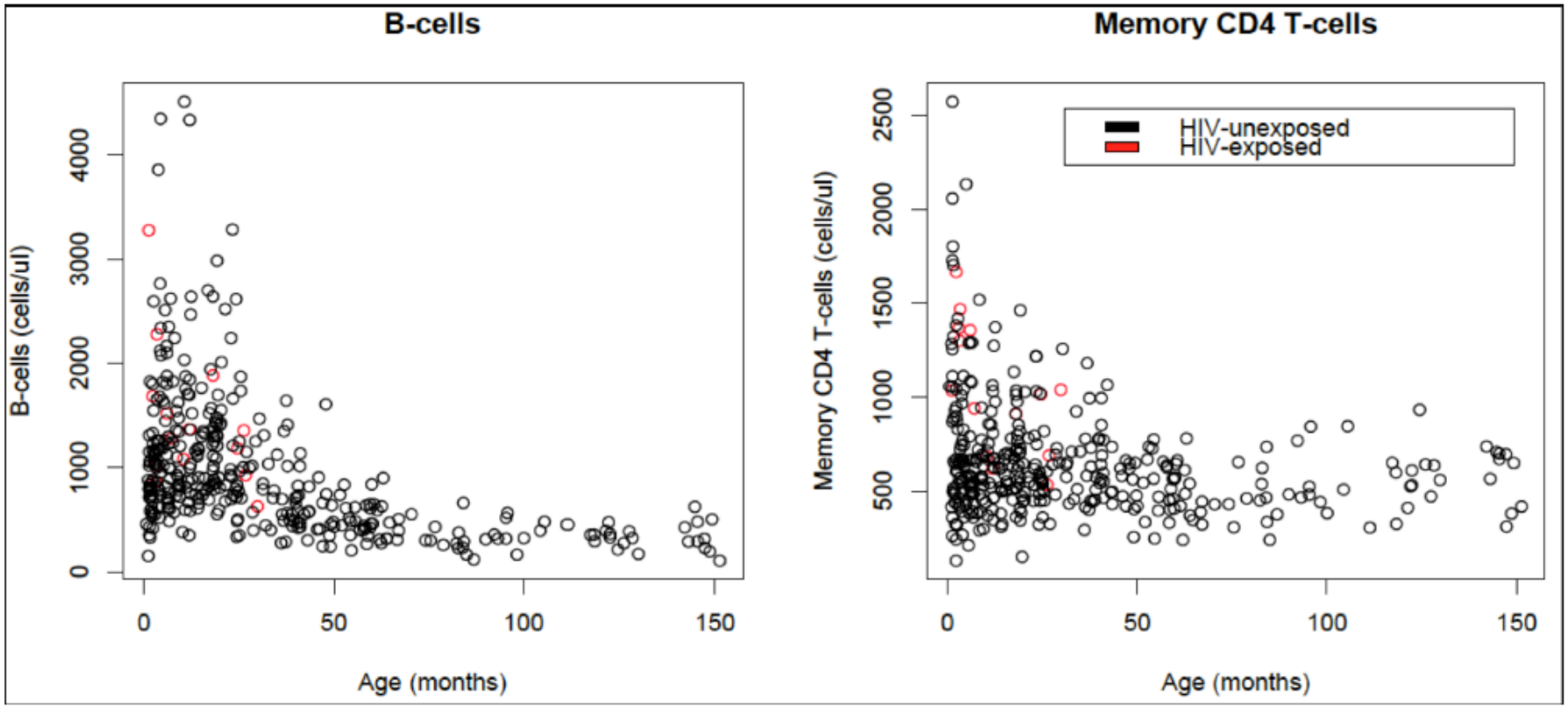
"Maternal conditions during pregnancy" was further divided to explore the effect of maternal HIV on the lymphocyte subsets (n=14). Differences were detected using the Wilcoxon rank sum test between these 14 children and the rest of the cohort with lower B-cells (CD19+HLADR+, $p=0.001$) and lower memory CD4 cells (CD3+CD4+CD45RO+, $p<0.0001$) in the HIV-unexposed children. However, removing them from the entire dataset did not affect the exponential regression curves at all, therefore the decision was made to include these children as part of this "healthy" population from the CWC (Figure 3.5).

Figure 3.4: Distribution of CD4 and CD8 in healthy children from the CWC including clinical subsets of conditions that might be presumed to influence the spread of data.



X-axis is age in months; y-axis is absolute cell counts in microlitre of blood for CD4 (left) and CD8 (right). Red circles = past history of a serious childhood illness e.g. TB, meningitis (n=11); Blue circles = acute recent illness within the past month but more than a week ago e.g. upper respiratory tract infections, gastroenteritis (n=69); Purple circles = maternal conditions during pregnancy e.g. TB, HIV, syphilis (n=28); Yellow circles = prematurity <32 weeks (n=13). Black lines represent the exponential regression curves delineated the best-fit, 95% and 5% of the data from the cohort.

Figure 3.5: The relationship between HIV-exposure and B-cells or Memory CD4 T-cells.



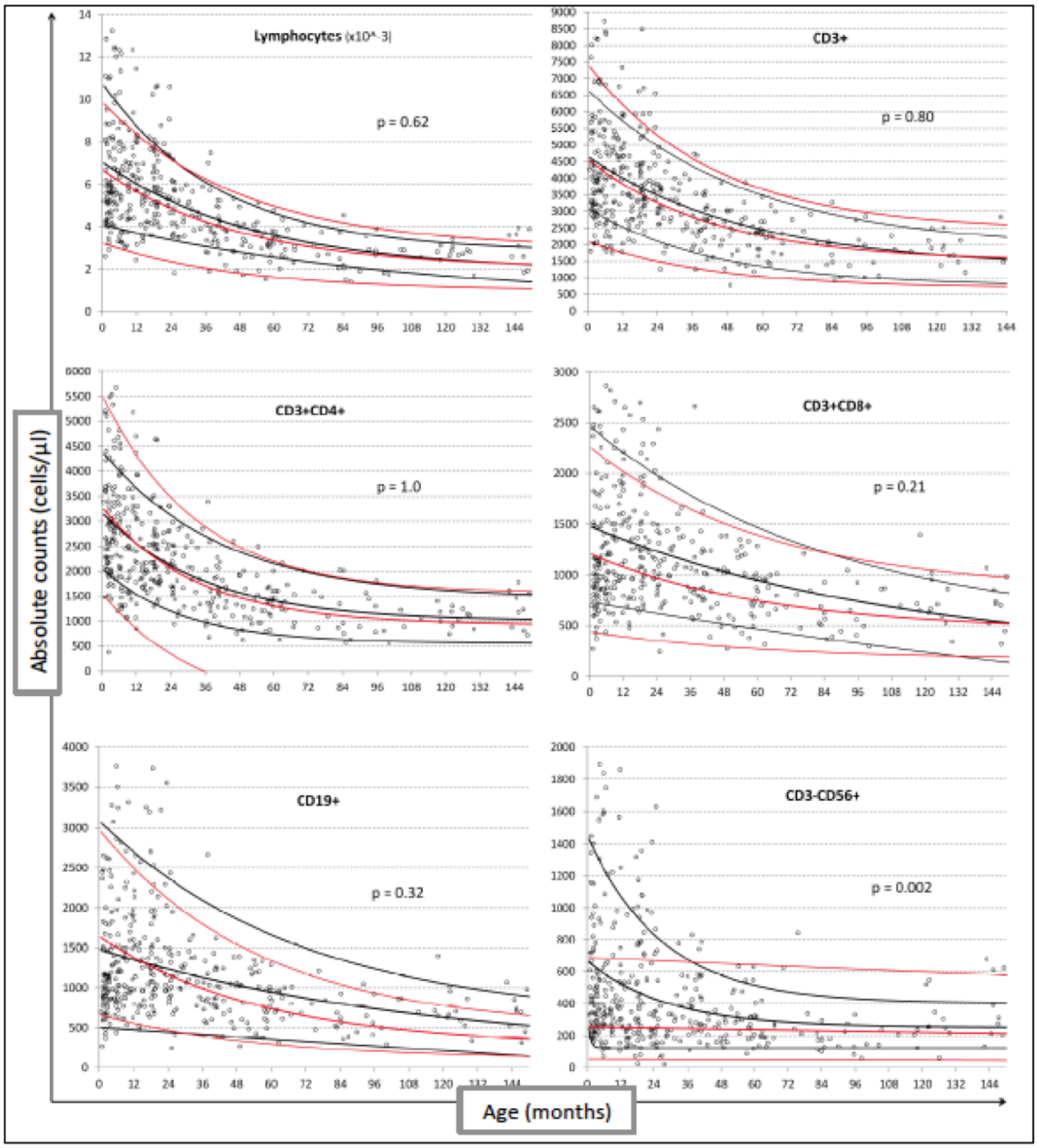
X-axis is age in months; y-axis is absolute cell counts in microlitre of blood. Statistical significance demonstrated using Wilcoxon rank sum test between HIV-exposed uninfected children (n=14) and the rest of CWC cohort (n=367) with lower B-cells (CD19+HLADR+, p=0.001) and lower memory CD4 T-cells (CD3+CD4+CD45RO+, p<0.0001) in HIV-unexposed children. Black circles reflect HIV-unexposed and red circles reflect HIV-exposed children.

Exponential regression curves between South African and German children

A selection of 6 graphs with fitted regression curves of common lymphocyte phenotypes: absolute lymphocyte count, CD3, CD4 and CD8 T-cells, B-cells (CD19+) and NK-cells (CD3-CD56+CD16+) are illustrated in Figure 3.6. Best-fit, 95% and 5% exponential regression curves are fitted for the CWC cohort (black lines) and Huenecke's data from healthy German children for comparison (red lines). Comparisons were made by assessing the difference between the central line of best-fit of the South African and German children by Wilcoxon Rank Sum. This was the most feasible analysis approach without access to the raw data of Huenecke's study. Differences by were found in NK-cells, activation markers (Figure 3.7), and particularly within the naïve (CD45RA+) and memory (CD45RO+) subsets. A trend towards higher absolute counts of CD8 T-cells was seen at a younger age in South African children compared to German children, and higher B-cells in the older childhood years.

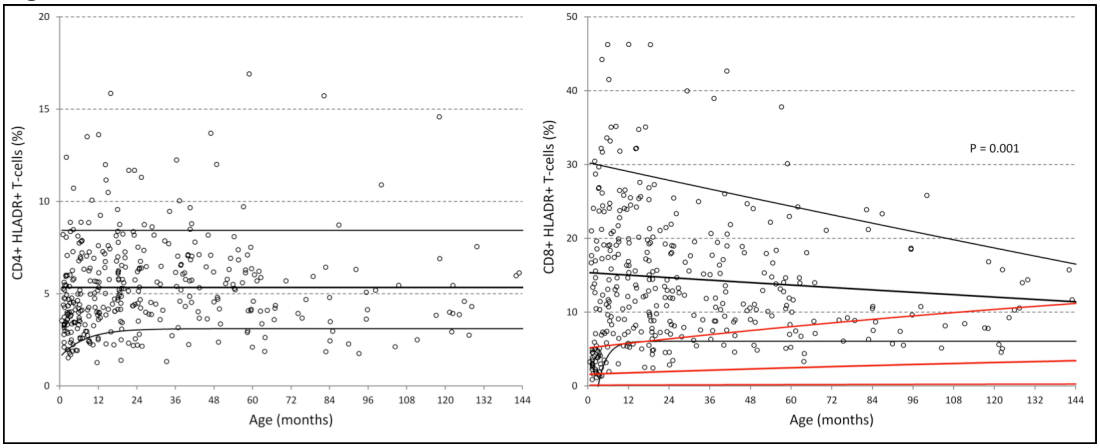
Marked differences were seen for both CD4 and CD8 T-cell memory populations particularly within the first 3 years of life, as illustrated by the change in naïve-memory ratios with age in Figure 3.8 (respectively p=0.07 and 0.01 overall). While Huenecke's data suggests naïve-memory ratios do not reach a 1:1 status [122] until the third or fourth decade of life, it is apparent that this is occurring by 11 years of age within this South African cohort.

Figure 3.6: Comparison of regression lines for six common lymphocytic markers between South African and German children.



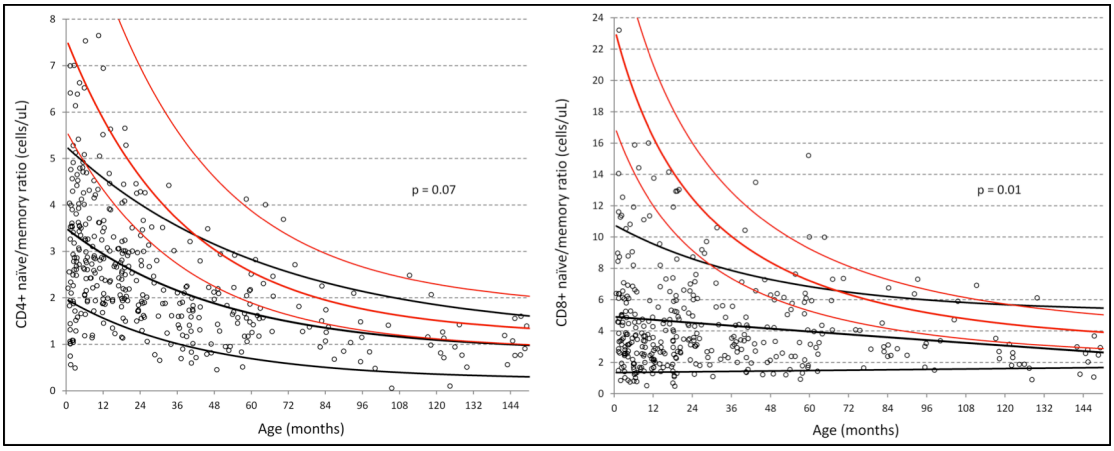
X-axis is age in months; y-axis is absolute cell counts in microlitre of blood. Black circles represent individual data-points from the South African cohort. Black lines represent the exponential regression curves delineated the best-fit, 95% and 5% of the data from the South African cohort. Red lines indicate best-fit, 95% and 5% of Huenecke's German data set [122]. From left to right, top to bottom: absolute lymphocyte count (cells/µl, p=0.6), T-cells (CD3+ cells/µl, p=0.8), T-helper cells (CD4+ cells/µl, p=1.0), cytotoxic T-cells (CD8+ cells/µl, p=0.2), B-cells (CD19+ cells/µl, p=0.3), and NK cells (CD56+ cells/µl, p=0.002). P-values represent the difference between the central line of best-fit of the South African and German children by Wilcoxon Rank Sum.

Figure 3.7: Activated subsets of CD4 and CD8 T-cells of South African children.



a) Percentage of CD4+HLA-DR+ of all CD4+ lymphocytes; b) Percentage of CD8+HLA-DR+ of all CD8+ lymphocytes compared to German children (p=0.001). X-axis is age in months; y-axis is absolute cell counts in microlitre of blood. Black circles represent individual data-points from the South African cohort. Black lines represent the exponential regression curves delineated the best-fit, 95% and 5% of the data from the South African cohort. Red lines indicate best-fit, 95% and 5% of Huenecke at al [122]'s data set. The p-value represents the difference between South African and German children by Wilcoxon Rank Sum. CD4+HLADR+ T-cells were not available from Huenecke's data set.

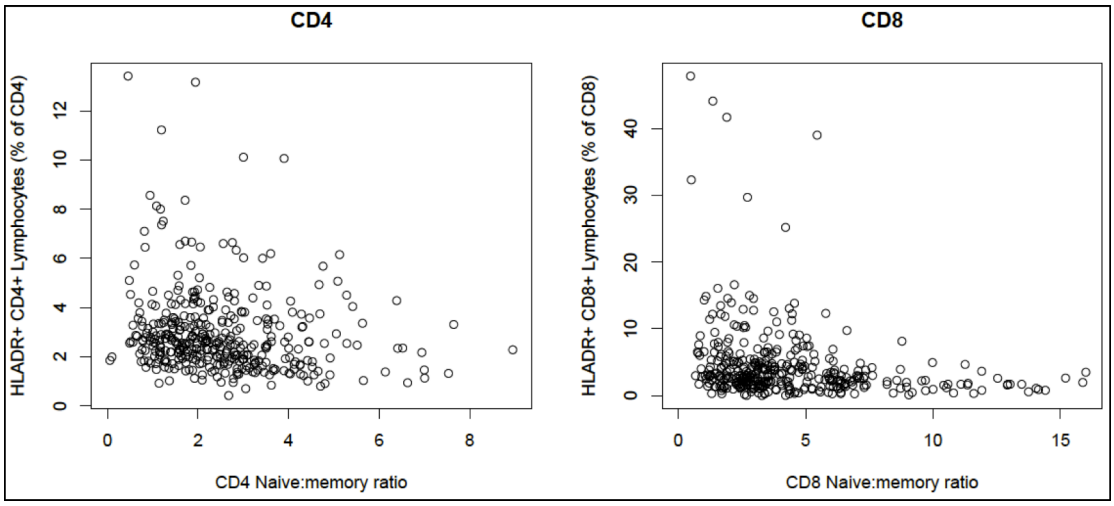
Figure 3.8: Comparison of naïve/memory ratios of CD4 and CD8 T-cells between South African and German children.



a) Ratio of naïve/memory CD4+ T-cells in cells/µl (left, p=0.07). b) Ratio of naïve/memory CD8+ T-cells in cells/µl (right, p=0.01). X-axis is age in months; y-axis is absolute cell counts in microlitre of blood. Black circles represent individual data-points from the South African cohort. Black lines represent the exponential regression curves delineated the best-fit, 95% and 5% of the data from the South African cohort. Red lines indicate best-fit, 95% and 5% of Huenecke at al [122]'s data set. P-values represent the difference between the central line of best-fit of the South African and German children by Wilcoxon Rank Sum.

Increased levels of activation of both CD4 and CD8 T-cells correlate positively with respective naïve/memory ratios. By simple linear regression there is a significant relationship between naïve/memory ratio and activation. For CD4 T-cells the parameters for linear regression are as follows: $F(1,379)=9.2$, $p=0.003$ with $R^2=0.02$; and the parameters for CD8 T-cells are: $F(1,378)=21.4$, $p<0.0001$ with $R^2=0.05$. For CD4 T-cells, predicted naïve/memory ratio is equal to 2.9-0.13(CD4+HLADR+ percentage of total CD4s), and for CD8 T-cells 5.0-0.13(CD8+HLADR+ percentage of total CD8s). This means that for every 1% increase in HLADR the naïve/memory ratio will decrease by 0.13 for CD4 T-cells and by 0.13 for CD8 T-cells (Figure 3.9). However, there appear to be children who do not fit this pattern, who have both low levels of activation and a naïve/memory ratio of less than one.

Figure 3.9: Relationship between naïve/memory ratios of CD4 or CD8 T-cells with levels of activated CD4 or CD8 T-cells.



X-axis reflects naïve/memory ratios calculated using the absolute cell count of the CD4 or CD8 T-cells naïve and memory subsets. Y-axis reflects percentage of activated CD4 or CD8 T-cells identified by the cell-surface activation marker HLA-DR. 381 samples were used from South African children from the CWC.

Age-categorised data between South African and US or Dutch children

The most significant finding when examining the distribution across all age groups (denoted by the overall p-value in Table 3.6) alongside the exponential regression curves were the increased proportions of CD4 and CD8 T-cells memory subsets across the entire age range of children from the CWC compared to their American and European counterparts. The CWC children also had lower CD4 and CD8 naïve subsets, particularly at less than 1 year of age. Both CD4 and CD8 T-cell naïve-

memory ratios appear to differ dramatically at less than a year of age, but not significantly so thereafter.

Additional differences were identified between lymphocyte subset distributions within individual age-categories in South African children versus those from North America and Netherlands. Significant differences found in the CWC cohort included: lower B-cells in children aged from 15 months to 13 years; higher percentage of CD8 T-cells in 10-16 year olds and higher activation of CD8 T-cells and T-cells overall in children below 15 months (Table 3.6).

Table 3.6: Differences in immunophenotype measurements within specific age groups between South African children and US (Shearer at al [103]) or Dutch children (Comans-Bitter et al [123]). A) Naïve-memory subsets. B) Activation markers and CD8 T-cells. C) B-cells. Information is displayed in the reference ranges of each data set. Measured data from the Child Wellness Clinic (CWC) has been calculated to match the age groups of the comparison studies. Reference ranges from Shearer et al are medians with 10^{th}-90^{th} centiles, and for Comans-Bitter et al are medians with 5^{th}-95^{th} centiles. Age-group comparisons were performed using the Chi-squared test and those with significant differences are highlighted in red: * indicates significance $p<0.05$, **$p<0.01$, *** $p<0.001$. Note: Shearer et al included 807 healthy children up until 18 years, although explicit numbers per age group were not published, each category included approximately 90 children except 3-6 months (81-89). The 'overall p-value' is the non-parametric Wilcoxon Rank Sum test for differences in the rank order of the median distributions between the South African study population and the comparison study in question i.e. Shearer et al or Comans-Bitter et al.

TABLE 3.6:	Age groups							Overall p-value
PART A: Naïve and memory subsets								
CD4+CD45RA+%	**0-3mths**	**3-6mths**	**6-12mths**	**1-2yrs**	**2-6yrs**	**6-12yrs**	**12+yrs**	
CWC	72.9 (51-83.8)*	78.4 (62.4-84.4)	73.9 (63.8-81.7)	72 (62.6-81.6)	63.8 (47-76.4)	54.7 (40.2-67.8)	48.5 (34.5-60)	0.25
Shearer	90 (64-95)	90 (77-94)	86 (64-93)	81 (63-91)	71 (53-86)	59 (46-77)	53 (33-66)	
CD4+CD45RO+%								
CWC	27 (16.2-48.9)***	22 (15.6-37.6)***	26 (18.3-36.2)***	28 (18.4-37.4)***	36.2 (23.6-53)***	45.3 (32.2-60)***	51.5 (40-65.5)***	0.01**
Shearer	10 (2-22)	8 (3-16)	9 (5-18)	12 (7-20)	16 (9-26)	21 (13-30)	28 (18-38)	
CD4+CD45RO+ cells/µl								
CWC	850 (320-1690)	640 (390-1290)	580 (390-1070)	630 (400-1030)	560 (380-780)	480 (320-740)	640 (400-730)	0.0006***
Shearer	320 (60-900)	330 (120-630)	340 (160-800)	400 (210-850)	360 (220-660)	350 (230-630)	380 (240-700)	
Naïve/memory CD4 ratio cells/µl								
CWC	2.7 (1-5.2)***	3.6 (1.7-5.4)*	2.8 (1.8-4.5)	2.6 (1.7-4.4)	1.8 (0.9-3.2)	1.2 (0.7-2.1)	0.9 (0.5-1.5)	0.18
Shearer	7.1 (4.1-20)	7 (5.9-10.8)	6.5 (4.6-6.9)	4.1 (3.4-4.8)	2.7 (2.3-2)	1.6 (1.6-1.4)	1.1 (1.1-1)	
CD8+CD45RA+%								
CWC	83.2 (65.9-92)	76.7 (63-89.8)**	77.1 (56.3-86.8)*	77.4 (62.6-89.3)	78.1 (62-88.2)	75.4 (64.2-87.1)	66.9 (52.2-77.1)	0.005**
Shearer	93 (80-99)	94 (85-98)	91 (75-97)	89 (71-98)	86 (69-97)	80 (63-92)	79 (61-91)	
CD8+CD45RO+%								
CWC	16.8 (8-34.1)***	23.3 (10-37.2)***	23 (13.2-43.7)***	22.6 (11-37.4)***	21.9 (11.8-38)***	24.6 (13-35.8)***	33.1 (23-47.8)***	0.002**
Shearer	3 (1-9)	3 (1-7)	3 (1-8)	6 (2-12)	9 (4-16)	12 (4-21)	13 (4-23)	
Naïve/memory CD8 ratio cells/µl								
CWC	5 (1.9-11.5)	3.3 (1.7-8.8)**	3.4 (1.3-6.6)**	3.4 (1.7-8.3)	3.6 (1.6-7.4)	3.1 (1.8-6.8)	2 (1.1-3.4)	0.25
Shearer	8.7 (4.5-15)	7.6 (4.8-18.3)	7.3 (4.5-12)	4.1 (3-8.2)	3.5 (2.5-4.2)	2.6 (2.3-4.4)	2.5 (2.3-4)	

PART B: Activation markers and cytotoxic T-cells									
CD8%	**1wk-2mths**	**2-5mths**	**5-9mths**	**9-15mths**	**15-24mths**	**2-5yrs**	**5-10yrs**	**10-16yrs**	
CWC	18.1 (10.7-28.9)	19.4 (11.8-28.6)	22.5 (12.1-31.7)	24.3 (13.3-34.6)	24.2 (13.8-34.9)	24.4 (15.7-35.6)	25.8 (15.1-34.8)	23.4 (18-30.8)**	0.13
Comans-Bitter	16 (9-23)	17 (11-25)	18 (13-26)	18 (12-28)	20 (11-32)	24 (14-33)	28 (19-34)	23 (9-35)	
CD3+HLA-DR+%									
CWC	4.7 (2.6-12.1)***	6 (2.6-20.1)***	8.5 (3.2-19.3)***	9.5 (3.7-19.2)***	9 (2.9-18.5)	8.4 (4.1-19.9)	6.9 (4.1-19.9)	7.5 (4-10.5)**	0.01**
Comans-Bitter	5 (1-38)	3 (1-9)	3 (1-7)	4 (2-8)	6 (3-12)	6 (3-13)	7 (3-14)	4 (1-8)	
CD8+HLA-DR+%	**0-3mths**	**3-6mths**	**6-12mths**		**1-2yrs**	**2-6yrs**	**6-12yrs**	**12+yrs**	
CWC	4.7 (2-20.6)	9.6 (2.5-31.7)***	16.9 (6.6-30.2)		14.4 (5.5-28.5)	12.5 (6-23.5)	8.8 (5.5-21.4)	11.7 (7.9-15.6)	1.0
Shearer	5 (2-20)	7 (3-17)	10 (4-27)		16 (6-33)	16 (7-37)	12 (6-29)	12 (5-25)	

PART C: B-cells									
CD19%	**1wk-2mths**	**2-5mths**	**5-9mths**	**9-15mths**	**15-24mths**	**2-5yrs**	**5-10yrs**	**10-16yrs**	
CWC	13 (8-21.1)	19.1 (9.7-31.4)	19.6 (12.6-38.3)	18.5 (10.6-29.1)	20.1 (10.6-32.6)*	15.8 (9.9-30.2)*	13 (9-18.4)*	11.8 (7.8-16.6)	0.05
Comans-Bitter	15 (4-26)	25 (14-39)	21 (13-35)	25 (15-39)	28 (17-41)	24 (14-44)	18 (10-31)	16 (8-24)	
CD19%	**0-3mths**	**3-6mths**	**6-12mths**		**1-2yrs**	**2-6yrs**	**6-12yrs**	**12+yrs**	
CWC	15.4 (8.8-21.8)	20.1 (12.6-29.6)	18.6 (11.9-27.8)		20 (12.9-30)	15.7 (11-24.1)	11.9 (9.4-16.7)*	11.5 (8.6-13.2)	0.16
Shearer	15 (6-32)	2 (11-41)	24 (14-33)		21 (14-33)	21 (14-33)	18 (13-27)	14 (6-23)	

Numbers	1wk-2mths	2-5mths	5-9mths	9-15mths	15-24mths	2-5yrs	5-10yrs	10-16yrs	
CWC, n	13	46	105	70	33	33	35	23	
Comans-Bitter, n	22	53	38	46	59	104	40	19	
Numbers	0-3mths	3-6mths	6-12mths		1-2yrs	2-6yrs	6-12yrs	12-18yrs	
CWC, n	45	40	54		79	118	37	8	
Shearer, n	~90	~90	~90		~90	~90	~90	~90	

3.2.4 Immunophenotyping from fresh and frozen cells

Preliminary work using fresh and frozen cells

The results in Table 3.7 indicate that no significant difference exists by t-test across all parameters explored comparing immunophenotypes between fresh and frozen PBMCs.

Table 3.7: Pilot data comparing immunophenotypes from 43 pairs of fresh and frozen PBMCs. *95% confidence interval that the true difference in means is not equal to zero.

Phenotype percentage	Fresh PBMCs	Frozen PBMCs	t-test p-value	95% confidence interval*
	Median [IQR]			
CD3+% of all lymphocytes	72.1 [68.2 – 82.1]	73.8 [66.3 – 81.7]	0.96	-3.98 to 4.17
CD4+% of all lymphocytes	36 [30.1 - 42]	37 [28 – 41.1]	0.96	-4.63 to 4.88
CD4+CD45RA-% of all lymphocytes	17.8 [14.1 – 20.7]	17.2 [10.4 – 19.5]	0.89	-3.35 to 3.86
CD4+CD45RA+% of all lymphocytes	17.7 [12.4 – 20.9]	17.1 [13.9 - 21]	0.92	-3.50 to 3.15
Ki67% of all CD3+ lymphocytes	2.1 [1.8 – 3.0]	1.7 [1.3 – 2.9]	0.37	-0.53 to 1.41
Ki67% of all CD4+ lymphocytes	2.3 [1.9 – 3.9]	2.2 [1.6 – 3.0]	0.99	-1.62 to 1.61
Ki67% of all CD4+ CD45RA- lymphocytes	3.8 [2.2 – 4.5]	2.7 [1.7 – 4.0]	0.43	-1.50 to 2.10
Ki67% of all CD4+ CD45RA+ lymphocytes	1.6 [1.0 – 4.1]	1.7 [1.0 – 3.8]	0.67	-2.11 to 1.38

There was no relationship between the percentage of live cells in the frozen aliquot and difference in lymphocyte subset measured compared to the expected value i.e. from the fresh PBMC aliquot. The percentage recovery of PBMCs and percentage of live cells has been recorded and presented in Table 3.8 for preliminary test-runs of 32 specimens each from the CWC and CHER. Of the CHER specimens, one had insufficient PBMCs and another had 100% dead cells after thawing therefore were excluded from subsequent analysis.

Table 3.8: Recovery of viable PBMCs from cryopreserved specimens. Pilot study of 30 specimens from CHER and 32 specimens from the CWC. Dead cells were identified by the viability marker efluor 660®, and live cells were therefore determined by flowcytometry by gated upon the unstained population of cells.

	Median [IQR]		
	% Cells recovered, counted manually	**% Dead, counted manually**	**% Live, by flowcytometry**
CHER, n=30	60.6 [42 – 86.1]	18.15 [5.9 – 24.2]	70.8 [56.9 – 80.4]
CWC, n=32	57.7 [26 – 85.2]	2.0 [0-6.5]	85.6 [75.2 – 88.8]

Although there is a difference between the manual count of live cells and percentage live cells by flowcytometry, there was a good correlation between counted dead cells and cell-viability (Pearson's correlation coefficient -0.62, p-value = 0.0001). The Clinical Laboratory Services cryopreservation protocol recommends 5-10 PBMCs x 10^6/ml of freezing media, however of the 32 CHER specimens processed, 9 were below the recommended concentration (minimum 1.4 x10^6/ml) and 2 above (maximum 1.0 x10^8/ml). 3 of the 11 suboptimal freezing concentrations had less than 60% viability, although in suboptimal low concentrations there was no correlation between freezing concentration and cell-viability in these 30 specimens (r = 0.03, p-value = 0.88).

Comparison of fresh whole blood assay with cryopreserved peripheral blood mononuclear cells (C-PBMCs)

To examine the effect of cryopreservation and methodological differences on immunophenotyping, 273 pairs of immune profiles as per Table 3.9 from the CWC (median age 1.4 years (range 0.05–12.3)) were compared: fresh whole blood assay (WBA, Methods section 2.4.3) against C-PBMCs (Methods section 2.4.2). In addition, 806 measures of CD4 from fresh WBA were available for comparison with CD4 measured from C-PBMCs from the same sample, from HIV-infected children from CHER on and off ART as per the trial ART-strategies [68].

Table 3.10 describes the differences between the fresh and frozen measures for each immunophenotype examined within each age group as used for the local reference ranges. The percentage change in the median between fresh to frozen is represented, along with the direction of change and whether the difference is statistically significant across all age-categories using Wilcoxon signed rank test. In all age groups, the median percentage of live cells thawed was greater than 82%. Almost all immune parameters demonstrated a significant difference between the fresh and frozen measures with the exceptions of CD3+ lymphocytes and CD4+

memory lymphocytes. Some parameters increased with the cryopreservation/thawing process (CD8+, CD19+, CD8+ memory and all activated lymphocyte subsets), whereas others decreased (CD3+, CD4+, naïve and memory CD4+, naïve CD8+ and CD56+ lymphocytes).

Table 3.9: The immunophenotypes used to compare lymphocyte subsets between the fresh whole blood assay and cryopreserved PBMCs.

Name of subset	Whole-blood assay	Cryopreserved PBMCs
CD3+ T-cells	CD3+	CD3+
CD4+ T-helper cells	CD3+/CD4+	CD3+/CD4+
CD8+ Cytotoxic T-cells	CD3+/CD8+	CD3+/CD8+
Naïve CD4+ T-cells	CD4+/CD45RA+	CD4+/CD45RA+
Memory CD4+ T-cells	CD4+/CD45RO+	CD4+/CD45RA-
Naïve CD8+ T-cells	CD8+/CD45RA+	CD8+/CD45RA+
Memory CD8+ T-cells	CD8+/CD45RO+	CD8+/CD45RA-
Activated CD3+ T-cells	CD3+/HLA-DR+	CD3+/HLA-DR+
Activated CD4+ T-cells	CD3+/CD4+/HLA-DR+	CD3+/CD4+/HLA-DR+
Activated CD8+ T-cells	CD3+/CD8+/HLA-DR+	CD3+/CD8+/HLA-DR+
CD19+ B-cells	CD19+/HLA-DR+	CD19+
Natural Killer cells	CD3-/CD56+	CD3-/CD56+

The differences between fresh WBA and C-PBMCs within lymphocyte subsets across age groups appeared fairly constant with the percentage of live thawed cells, CD3+ and CD4+ lymphocytes, CD4+ naïve and CD56+ lymphocytes. CD4+ memory and CD8+ naïve lymphocytes had no particular pattern of differences across the age groups, with differences appearing to be quite erratic. Whereas in other lymphocyte subsets there appeared to be more of a pattern: the difference between fresh and frozen generally increased in all activated subsets (CD3+HLADR+, CD4+HLADR+ and CD8+HLADR+) with age; and in B-cells the difference between fresh and frozen was much larger in children over 2 years of age. These different patterns of change between fresh and frozen measurements of the same parameters are illustrated in Figure 3.10.

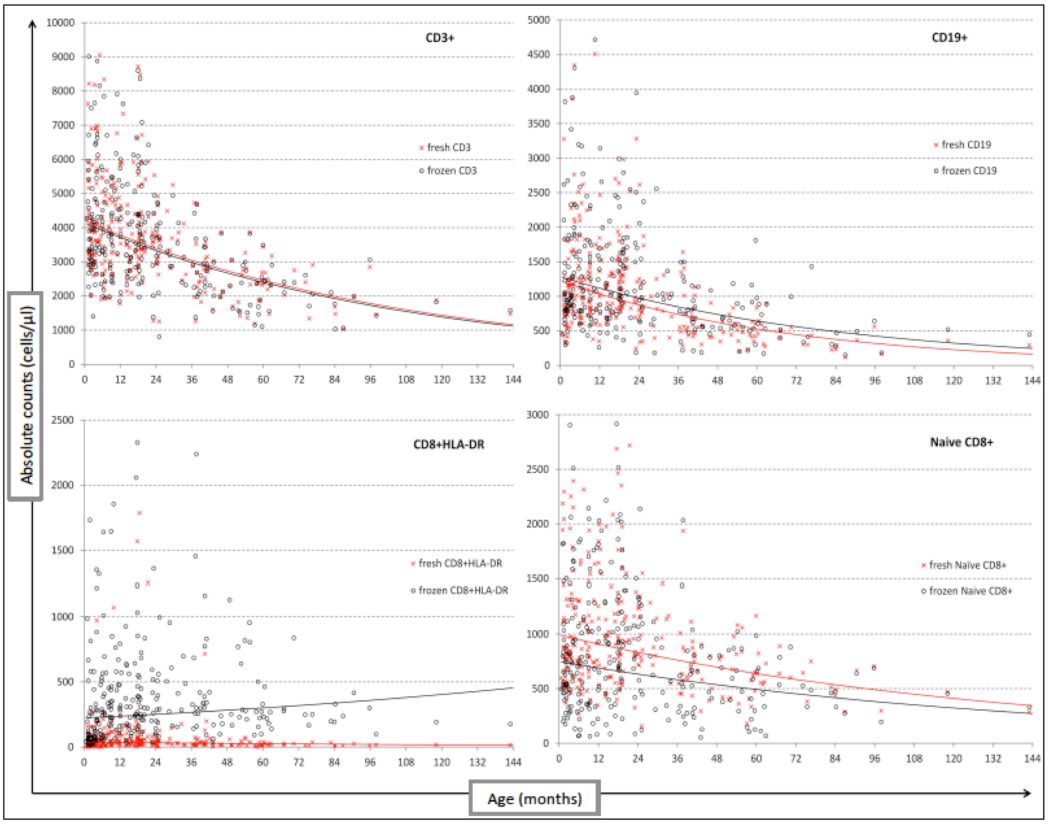
Table 3.10: Comparison of immunophenotypes between fresh whole blood assay versus cryopreserved PBMCs. Results in absolute cell count $x10^9$/dL of blood (Median and 95% confidence interval (CI)). *p-values represent the significance of the difference between fresh and frozen across all age-groups and were determined by Wilcoxon Signed Rank Test.
[t]Mean % and direction of change from fresh to frozen are reported.

TABLE 3.10: Cell group (Median cells x10^9/dL with 95% CI)	Age ranges 0-3 months (N=36)	3-6 months (N=32)	6-12 months (N=43)	1-2 years (N=69)	2-6 years (N=79)	6+ years (N=14)	*p-value	Direction of change & †Mean % change
Total fresh lymphocytes	5.5 (3.7-11.1)	6.3 (3.5-12.5)	6.0 (3.2-9.4)	5.7 (3.5-10.6)	3.9 (1.9-6.5)	2.6 (1.4-3.9)		
Live percentage (following freezing)	89.8 (44-95.9)	89.4 (58.1-95.6)	88.1 (53.2-96.1)	88.7 (49.1-95.3)	82.8 (49.8-94.6)	85.7 (60-92)		Down
% Change in median	-10.3	-10.7	-11.9	-11.3	-17.2	-14.4		-12.6
CD3+ fresh	3.6 (2.6-7.6)	4.1 (2.6-8.2	4.2 (2.0-5.9)	3.8 (2.1-6.7)	2.8 (1.4-4.7)	1.9 (1.0-2.9)		
CD3+ frozen	3.5 (2.0-7.5)	4.1 (2.4-8.2)	3.9 (2.0-6.7)	3.8 (2.1-7.1)	2.7 (1.4-4.7)	1.9 (1.0-3.1)	0.97	Down
% Change in median	-1.6	1.7	-6.1	1.3	-2.4	-0.2		-1.2
CD4+ fresh	2.6 (1.7-5.2)	2.9 (1.7-5.5)	2.5 (1.3-4.1)	2.2 (1.3-4.6)	1.6 (0.8-2.9)	1.1 (0.6-1.8)		
CD4+ frozen	2.3 (1.2-5.0)	2.8 (1.2-5.8)	2.3 (1.0-4.2)	2.0 (1.0-4.0)	1.4 (0.5-2.7)	1.1 (0.4-1.9)	<0.01	Down
% Change in median	-11.0	-5.8	-7.5	-7.0	-12.9	3.6		-6.8
CD8+ fresh	1.0 (0.6-2.6)	1.1 (0.5-3.1)	1.3 (0.6-2.8)	1.3 (0.6-3.2)	1.0 (0.4-1.7)	0.7 (0.4-1.2)		
CD8+ frozen	1.0 (0.4-2.4)	1.4 (0.7-3.4)	1.4 (0.7-2.8)	1.6 (0.6-3.9)	1.0 (0.4-1.8)	0.6 (0.3-0.9)	<0.01	Up
% Change in median	3.4	24.9	15.4	26.4	0.3	-9.4		10.2
CD19+ fresh	0.8 (0.4-1.8)	1.3 (0.7-3.9)	1.0 (0.6-2.3)	1.2 (0.6-2.6)	0.6 (0.3-1.6)	0.3 (0.2-0.6)		
CD19+ frozen	0.9 (0.3-2.7)	1.4 (0.7-3.9)	1.1 (0.5-2.8)	1.3 (0.5-2.8)	0.7 (0.2-1.8)	0.5 (0.1-1.4)	<0.01	Up
% Change in median	17.7	11.2	11.8	9.1	28.4	55.0		22.2
CD3+HLADR+ fresh	0.2 (0.09-0.9)	0.3 (0.1-1.7)	0.4 (0.1-1.4)	0.4 (0.1-1.1)	0.2 (0.08-0.8)	0.1 (0.07-0.2)		
CD3+HLADR+ frozen	0.2 (0.07-1.2)	0.2 (0.09-1.5)	0.5 (0.2-2.2)	0.5 (0.2-2.7)	0.5 (0.2-1.6)	0.3 (0.2-0.5)	<0.01	Up
% Change in median	-0.3	-23.7	24.1	29.1	108	113		41.8

TABLE 3.10: Cell group (Median cells x10^9/dL with 95% CI)	Age ranges 0-3 months (N=36)	3-6 months (N=32)	6-12 months (N=43)	1-2 years (N=69)	2-6 years (N=79)	6+ years (N=14)	*p-value	Direction of change & [†]Mean % change
CD4+HLADR+ fresh	0.07 (0.03-0.2)	0.07 (0.01-0.2)	0.05 (0.03-0.2)	0.06 (0.03-0.1)	0.05 (0.02-0.1)	0.03 (0.01-0.05)		
CD4+HLADR+ frozen	0.1 (0.05-0.3)	0.1 (0.03-0.5)	0.1 (0.04-0.4)	0.1 (0.05-0.3)	0.1 (0.05-0.4)	0.07 (0.04-0.1)	<0.01	Up
% Change in median	43.3	39.7	109	93.7	190	123		99.6
CD8+HLADR+ fresh	0.01 (0.003-0.2)	0.05 (0.002-0.3)	0.05 (0.01-0.2)	0.05 (0.01-0.2)	0.03 (0.004-0.2)	0.02 (0.009-0.04)		
CD8+HLADR+ frozen	0.07 (0.02-1.0)	0.1 (0.05-0.1)	0.3 (0.07-1.6)	0.3 (0.08-2.1)	0.3 (0.09-1.1)	0.2 (0.1-0.4)	<0.01	Up
% Change in median	393	220	608	504	1000	1329		676
CD4+CD45RA+ fresh	1.8 (0.7-4.1)	2.4 (1.4-4.1)	1.8 (0.8-3.1)	1.6 (0.9-3.2)	1.0 (0.4-2.0)	0.5 (0.3-1.2)		
CD4+CD45RA+ frozen	1.6 (0.6-4.3)	1.7 (0.4-5.0)	1.1 (0.3-3.4)	1.3 (0.4-2.4)	0.7 (0.3-2.0)	0.7 (0.2-1.2)	<0.01	Down
% Change in median	-14.9	-28.8	-41.0	-18.3	-26.2	34.3		-15.8
CD4+CD45RO+ fresh	0.9 (0.3-1.8)	0.7 (0.3-1.5)	0.6 (0.4-1.3)	0.6 (0.3-1.2)	0.6 (0.3-1.0)	0.5 (0.3-0.8)		
CD4+CD45RO+ frozen	0.6 (0.3-2.1)	0.6 (0.3-1.8)	0.6 (0.3-2.2)	0.6 (0.4-1.8)	0.5 (0.2-1.2)	0.4 (0.2-0.7)	0.21	Down
% Change in median	-31.2	-9.8	3.3	-1.1	-6.3	-21.9		-11.2
CD8+CD45RA+ fresh	0.8 (0.5-2.2)	0.9 (0.5-2.3)	0.9 (0.4-1.7)	1.0 (0.5-2.4)	0.7 (0.3-1.3)	0.5 (0.3-0.8)		
CD8+CD45RA+ frozen	0.6 (0.2-1.8)	0.8 (0.1-2.5)	0.7 (0.08-1.9)	0.9 (0.2-2.1)	0.6 (0.1-1.3)	0.5 (0.2-0.7)	<0.01	Down
% Change in median	-30.6	-5.6	-16.3	-3.5	-21.4	-5.0		-13.7
CD8+CD45RO+ fresh	0.2 (0.05-0.7)	0.3 (0.08-1.1)	0.3 (0.08-0.8)	0.3 (0.07-1.2)	0.2 (0.08-0.6)	0.2 (0.07-0.5)		
CD8+CD45RO+ frozen	0.4 (0.08-1.2)	0.5 (0.2-1.5)	0.6 (0.1-2.3)	0.5 (0.2-1.6)	0.3 (0.08-1.1)	0.1 (0.06-0.5)	<0.01	Up
% Change in median	121	95.2	77.6	67.9	70.6	-22.7		68.2

TABLE 3.10: Cell group (Median cells x10^9/dL with 95% CI)	Age ranges 0-3 months (N=36)	3-6 months (N=32)	6-12 months (N=43)	1-2 years (N=69)	2-6 years (N=79)	6+ years (N=14)	*p-value	Direction of change & [1]Mean % change
CD56+ fresh	0.4 (0.2-1.5)	0.4 (0.1-1.7)	0.4 (0.1-1.6)	0.4 (0.1-1.4)	0.3 (0.1-0.8)	0.2 (0.06-0.8)		
CD56+ frozen	0.4 (0.1-1.7)	0.4 (0.2-1.5)	0.3 (0.1-1.1)	0.3 (0.1-1.0)	0.3 (0.1-0.8)	0.2 (0.08-0.4)	<0.01	Down
% Change in median	-19.2	-4.5	-20.3	-21.7	3.5	-25.0		-14.5

Figure 3.10: Regression curves to illustrate the variability of the difference between fresh and frozen measures of common immunophenotypes from birth to 12 years old.



X-axis is age in months, and y-axis is absolute cell count in cells per microlitre of blood. Red data-points reflect fresh values and black reflects frozen data. The regression curves, calculated by Dr Martin Nieuwoudt, were estimated separately for fresh and frozen data. 273 pairs of data were examined and all children had both sets of fresh and frozen values. A) CD3+ lymphocytes (no significant difference between fresh and frozen). B) CD19+ lymphocytes (overall significantly different). C) CD8+HLADR+ lymphocytes (increasingly significant difference with ageing). D) Naïve CD8+ lymphocytes (decreasing significant difference with ageing).

Further factors that might influence the integrity of thawed subsets were explored including gender, recent illness and haematological components of blood. No relationship between gender and the difference between measures of WBA and C-PBMCs within each subset were identified. Monocyte and lymphocyte percentage had a significant positive correlation with the difference between fresh and frozen in activated CD3 and CD4 subsets (p-values all <0.04) i.e. the higher the white blood cell percentage, the less the difference between WBA and C-PBMCs; however the spread of data was wide (R^2 all <0.07) therefore the relationship is unlikely to be linear and directly related to white blood cell subset. In contrast granulocyte and eosinophil percentage had a negative correlation with activated CD3 and CD4

subsets (p-values all <0.04) i.e. the higher the white blood cell percentage, the greater the difference between WBA and cryopreserved PBMCs, yet again R^2 is low. All other lymphocyte subsets explored did not demonstrate an association with any of the haematological components available from routine full blood count.

The CWC recorded whether the child participant had a recent illness, defined as between 1-4 weeks ago (n=44, 16%). Comparing differences in WBA and cryopreserved PBMC values between those who had a recent illness and those who did not, revealed the difference in WBA and cryopreserved PBMCs to be significantly less in naïve and memory CD8 T-cells (p=0.0001 and p<0.0001 respectively), and naïve CD4 (p=0.01) in those with recent illness.

Effect of HIV/ART on differences in fresh and frozen immunophenotypes

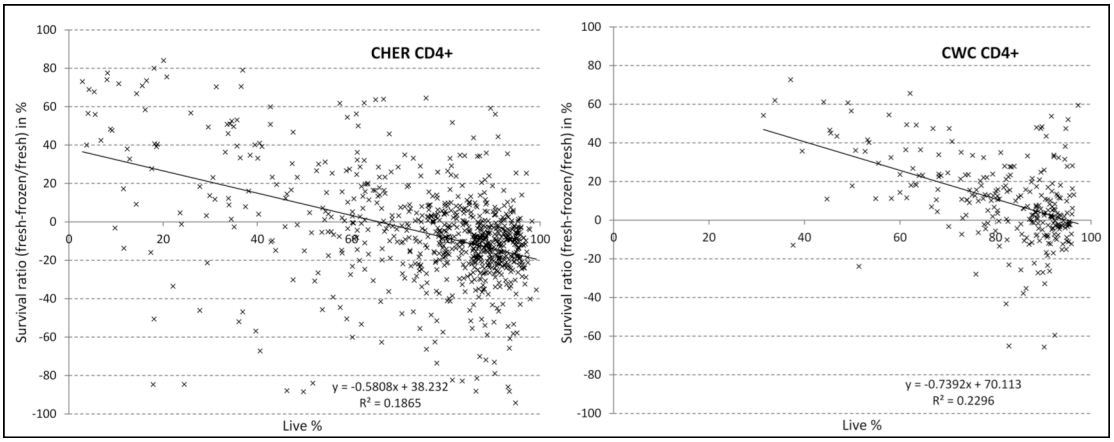
To explore the differences between immunophenotypes measured from a fresh WBA and cryopreserved PBMCs in HIV-infected children, 806 pairs of CD4 measurements were examined from participants of the CHER trial. The difference in CD4 cells between fresh and frozen was a median of 2.5% higher in frozen cells (IQR -1.7 to 6.2%, range: -41.7 to 30.4%). This was not largely dissimilar to the differences seen in the CWC healthy child cohort, median 2.9% lower in frozen (IQR -8.9 to 1.3%, range: -34.3 to 19.8%). No significant relationship exists by logistic regression between the age at which the sample was taken and difference between fresh WBA and cryopreserved PBMCs for CHER (OR 1.0 [95% CI: 1.0-1.0] or for CWC (OR 1.1 [95% CI: 0.6-1.8]. No relationship was seen between CHER trial arm and the difference between CD4 percentage by fresh WBA versus cryopreserved PBMCs. However if a child had reached a trial-defined endpoint (i.e. immunological, clinical, or virological failure of first-line ART or death) they were more likely to have a significant difference between fresh WBA and cryopreserved PBMCs (OR 7.1 [95% CI 1.7-29.1], p=0.007) than those who did not reach a trial endpoint.

The role of percentage of live thawed PBMCs

Whether the percentage of PBMCs that died during the cryopreservation-thaw processes might determine the integrity of thawed subsets was explored by examining the percentage of live thawed PBMCs with the differences between fresh and frozen lymphocyte subsets in healthy children, and also the CD4 measures available from the CHER trial. As described in Methods section 2.5, the difference between fresh and frozen was quantified as a survival ratio in cells/μl (i.e. [fresh WBA

– frozen PBMCs] / fresh WBA). However, in neither CWC nor CHER were any clear relationships evident between the live percentage and the survival ratio of each lymphocyte subset. This was true when examining all CHER data together and the arms separately. Although the data is widespread, in frozen specimens with less than 50% live cells, there appears to be a greater "survival ratio" i.e. when there are >50% dead cells it appears more likely that the frozen cell count would over-estimate the true fresh cell count (Figure 3.11).

Figure 3.11: Relationship between the percentage of live thawed PBMCs and CD4 survival ratio.



A) All CHER data. B) All CWC data. The x-axes represent the percentage of live cells. The y-axes of both graphs represent the survival ratio ("[fresh-frozen]/fresh" = cells/µl of WBA subtracted from cell/µl of cryopreserved PBMCs, then divided by cells/µl of WBA).

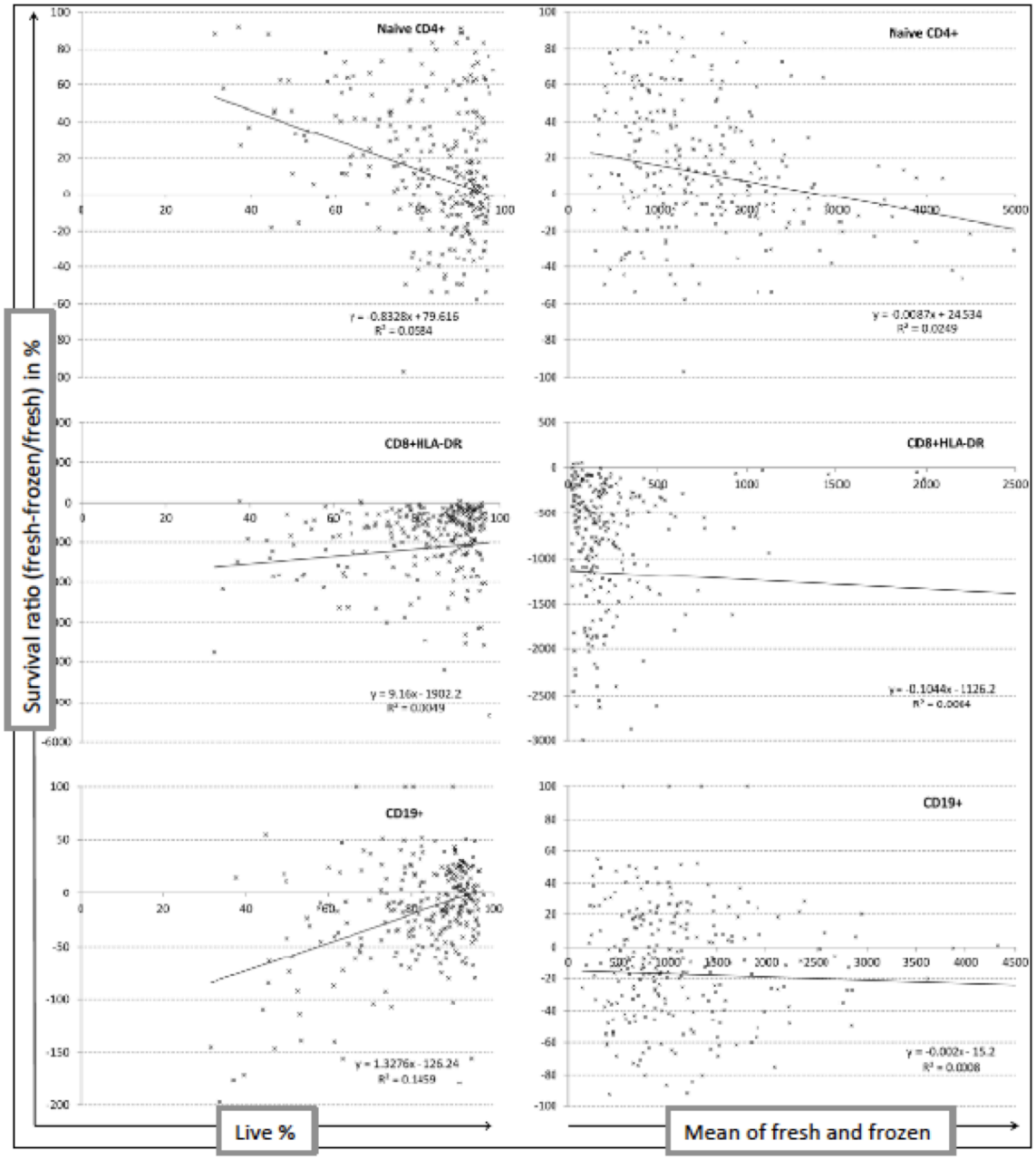
This relationship was further investigated using two approaches: linear regression between percentage of live thawed PBMCs against the survival ratio, and Bland-Altman plots comparing the mean of fresh and frozen against the survival ratio (illustrated in Figure 3.12). Both formats demonstrate a wide spread of data (R^2 at best was 0.2 for CD4+ T-cells), making it difficult to determine any particular pattern other than making a general observation that for most lymphocyte subsets, a greater proportion of dead cells appears to decrease the accuracy of the results from the cryopreserved specimens.

Use of fresh "anchor" values in research on cryopreserved PBMCs

Finally, the utility of having a fresh whole blood measure as an "anchor" value for the rest of the immune profile obtained from C-PBMCs was explored i.e. if the fresh CD4 value did not differ significantly from the frozen measure of CD4, would this indicate that other immunophenotypes measured from frozen would also not differ

significantly? This was explored using CD3, CD4 and CD8 as the anchor marker in the CWC data set, however no relationship was identified between the comparability of the anchor marker and the comparability of the rest of the immunophenotypes examined.

Figure 3.12: Relationship between percentage of live thawed cells and difference between fresh whole blood assay (WBA) and cryopreserved PBMCs illustrated by three lymphocyte subsets: naïve CD4 T-cells, activated CD8 T-cells and CD19 B-cells.



The y-axes of all graphs represent the survival ratio ("fresh-frozen/fresh" = cells/µl of WBA subtracted from cell/µl of C-PBMCs divided by cells/µl of WBA). The x-axes of the 3 graphs to the left represent the percentage of live cells, and the 3 graphs to the right are Bland-Altman plots, therefore the x-axes of these graphs are the mean of the WBA and C-PBMCs.

3.3 Discussion

3.3.1 Data from the Child Wellness Clinic

A substantial number of specimens and supporting data were obtained from the Child Wellness Clinic. The distributions of maternal age and Z-scores for the child's weight were approximately normally distributed indicating that this sample population may be a fair representation of the CWC population, although it was normally distributed around -1 to 0 Z-score for weight-for-age suggesting the CWC population was of a lower weight-for-age than the reference population. The proportions of antenatal events, neonatal events and medical history of note are not atypical for an informal settlement in South Africa according to clinicians with experience working in the region. Clinic attendance was voluntary and might have led to selection bias, however the participants' parents appeared to come from a variety of backgrounds and had different motivations for participation. The majority of children were recruited opportunistically as they attended for routine paediatric care. Others learned about the clinic through word of mouth and desired a paediatrican's "check-up" and more specifically, the blood test to "check everything was okay". The HIV-test itself was also motivation for a few, as was the philanthropic belief that their child would take part in research that could potentially benefit other children, especially those infected with HIV. There was a proportion from poorer home-environments whose attendance was clearly driven to acquire the nutritional supplements and food voucher. Often in these cases, the mother and child were not accessing routine care, and it was an opportunity to engage the mother with the clinic's services and get the children up-to-date with vaccinations, vitamin drops and de-worming.

There may have been selection bias introduced by the classification of the CWC participants' ethnicity. In South Africa, the ethnicity referred to as "coloured" implies heterogeneous ancestry from Europe, South Asia or Indonesia with a native Khosian or Bantu tribes of Southern Africa, although it could also indicate any degree of admixture. The region of the Western Cape is known to have the highest levels of mixed ancestry in the world, however genetic contribution to the coloured population of this region has been found to come mostly from Khosian populations [132] and therefore the distinction between whether an individual is of pure Khosian descent ("black") or distant mixed ancestral descent ("coloured") may not always appear obvious. It is also worth noting the history of "imposed ethnicity" during the apartheid era [133], and reports that several Indian and indigenous African groups chose to be

classified as “coloured” since there were certain advantages over being classified as “black” [134]. The South African assistant employed to help with recruitment, translation and the running of the CWC guided the distinction of ethnicity, 22% black and 78% coloured. Therefore without formal genetic differentiation, analysis of the haematological and immunological parameters or thymic output with regards to ethnicity is limited by the system of classification used in this study. While maternal age and socioeconomic status are comparable to the majority of the South African population for the purpose of the local reference ranges study, and to the CHER participant demographics for the thymic output comparisons, ethnicity does differ as 79% of the South African population [135] and 96% of the CHER participants are classified as black, and this will be a limitation of any comparisons made with the CWC.

3.3.2 International comparison of lymphocyte subsets

As health provision improves and molecular diagnostics make diagnosis more cost effective and accessible in sub-Saharan Africa, the number of patients receiving treatment for HIV and other infectious diseases, as well as the number of clinical trials undertaken is expected to increase substantially. Although there are published studies detailing reference ranges for children in African countries [118, 120], particularly for children with HIV [117], the dearth of local paediatric reference range data in South Africa [136] prompted this substudy to establish a relevant local set laboratory reference values to ensure that health care, treatment and monitoring is appropriate for the population of children being assessed. These reference ranges have since been published by South African Medical Journal (Appendix XVI, [98]). The data generated should be representative of lymphocyte subsets in these children living in impoverished communities who are more likely to be exposed to a significant diseases, such as TB and HIV, than their counterparts in industrialised countries.

The main finding of the international comparison substudy was the dramatic differences in the ratio of naïve to memory T-cell populations occurring in the South African children studied here. Parity between these populations of cells was reached some 20-30 years earlier than observed in European studies [103, 122, 123]. This has been noted before [137, 138]; and may be explained by a reduction in thymic output with depletion of the naïve T-cell pool and/or accompanied by expansion of memory cells as naïve T-cells encounter antigen and memory populations proliferate. Until now, characterisation of this transition throughout the first decade of life has not

been described, nor compared across continents where genetics, nutrition and environmental antigenic exposure differ extensively.

Increased proportions of CD8 T-cells, natural killer cells and activated T-cells were demonstrated in healthy South African children compared to their US or European counterparts. Increased CD4 or CD8 activation was indeed associated with decreasing naïve/memory ratio. These findings contribute to the explanatory hypothesis that environmental exposure to common pathogens such as herpesvirus, cytomegalovirus and Epstein-Barr virus; alongside microbial translocation, may drive the switch from naïve to memory T-cells. The burden of poverty undoubtedly plays a role in the abundance of environmental pathogens [139], and combined with poor nutritional status is likely to affect rates of microbial translocation [140] and even immune responses to such pathogens [141, 142].

The burden of acquired immunodeficiency, such as paediatric HIV infection, lies in Sub-Saharan Africa, whereas the vast majority of primary immunodeficiencies and allergic conditions appear to occur in industrialised countries. This may simply be due to availability of advanced clinical care and diagnostics, however it might also be related to evolution of the paediatric immune system within an environment deficient in immunological stimulus. It is conceivable that such environmental pathogen-stimulation and subsequent early naïve-to-memory transition is not only a typical process, but also prerequisite for the immune system to establish a repertoire of functional immune responses. Darwin's evolutionary theory argues that all life comes from the same ancestral source, and that diversity and survival occur through a branching pattern of evolution by adaptation to our changing environment [143]. Whether the differences we have observed represent an environmental adjustment or an evolutionary change in the paediatric immune system cannot be answered by this data, yet it presents the intriguing question as to which paediatric immune system is more evolutionarily normal.

Our data is consistent with several studies in African settings showing that early environmental exposure to infection can program human immune responses [144-146] reinforcing the *Hygiene Hypothesis* which suggests that infections in early life may prevent development of pathological immune responses to allergens and autoallergens [147].

It is not possible to determine whether differences seen are related to environmental exposure or genetics. The European studies for comparison do not describe ethnicity, and although the US study reports the majority of their cohort to be of African-American race (58%), the genetics may be quite different from an African population. While our cohort broadly represents the general population of South Africa in terms of socio-economic background, it does not represent approximately 25% of the South African population that are relatively wealthier with well-equipped and sanitised home and school environments [135], and thereby might have comparatively less disease exposure and presumably different "normal" immunological phenotypes compared to the participants of the CWC.

The inclusion of the children with histories of significant illnesses, maternal infections during pregnancy, recent illnesses and prematurity less than 32 weeks gestational age in the CWC healthy cohort might be a source of debate. However, since the prevalence in the CWC cohort is not atypical compared to the study population and there is no clear biological evidence to implicate the effect of these conditions on the child's developing immune system it may be acceptable to include these conditions. The sub-analyses performed on the 14 children who were born to HIV-infected mothers did not affect the overall regression curves in this study due to the small number and age distribution of the group. A larger study might be warranted to explore these potential differences in more detail, especially since only 3.7% of children in our cohort were HIV-exposed compared to recent estimates of 32% of infants born in the public sector in South Africa [40]. This low rate of HIV-exposure might be explained by the fact that the clinic was promoted as a "healthy child" clinic where a HIV test would be done on all children, and this may have deterred mothers who knew they were HIV-infected.

There are numerous limitations to the outcomes of the comparison of lymphocyte subsets between South African children and the three studies in children from the US and Europe. These three cohorts come from contrasting environments to South Africa, and the data is generated from studies that used non-identical methodologies. There are multiple practical factors that might influence data derived from flowcytometric studies including sample transport, storage and preparation, choice of fluorochrome-conjugated antibodies and immunological markers to define subsets of interest. This makes direct comparison between such studies challenging. In an attempt to minimize the affect of these factors, exponential regression curves were used to compare the changes in immunological parameters across the age-range

examined, as this approach should help to reduce the influence of confounding factors.

One of the limitations of this study is that the mathematical method applied to create exponential curves from these data does not account for the possibility that cell counts may increase and peak during the first year of life with subsequent decline, however ongoing work by my collaborators will further explore this issue.

There is potential for multiple statistical comparisons, as those that were done and represented in Table 3.6, to lead to significant differences detected that might be due to chance rather than biological plausibility. These calculations were not adjusted for multiple comparisons, therefore a degree of caution should be applied in the interpretation of any significant finding that was not replicated by other analyses across the studies examined. A combination of statistical approaches have been applied to the analysis of these data-sets, however regardless of the statistical approach employed, the main findings of rapid and early transition of naïve T-helper and cytotoxic T-cells to their respective memory populations in the CWC, was concordant across all 3 of the compared international studies. This seems most likely due to the induction of activation markers by increased exposure to environmental pathogens as seen in the South African study population.

There were two main aims of setting up the CWC. The first was to acquire research specimens to establish and explore what may be normal thymic output and enable comparison with thymic output measured from the CHER trial (Chapter 5). The second was to collect immunological data from healthy South African children to establish local reference ranges. These immunophenotypes measured have also been used for comparison with immunophenotypes from HIV-infected children on different ART-strategies (Chapter 4).

In South Africa clinicians and laboratories currently use a combination of the reference ranges published by Comans-Bitter et al and Shearer et al where children from better socioeconomic backgrounds comprise the study groups. A number of important differences between the CWC and international studies were found, highlighting value of having contextually appropriate reference ranges available. Although no gross difference was identified with the lymphocyte markers most commonly used in clinical practice such as CD4, CD8 and CD4/CD8 ratios, a dramatic and significant difference was demonstrated in the rapid early decline of the

naïve-memory ratios of both CD4 and CD8 T-cells alongside increased lymphocyte activation in this paediatric population. While providing valuable insight into the developing paediatric immune system within an African context, the long-term health implications of these findings certainly require further investigation.

3.3.3 Immunophenotyping from fresh and frozen cells

This substudy aimed to outline the differences between immunophenotypes measured by fresh whole blood assay and cryopreserved PBMCs. A number of significant differences were observed, and where possible the causes were explored. While some differences may be directly related to the effect of cryopreservation, there are a number of methodological differences that need to be taken into account.

The direct comparison of fresh and frozen PBMCs done, as preliminary work did not reveal any significant differences between the parameters investigated. However, in the much larger study of healthy children from the CWC, most immune parameters differed to some extent between whole blood assay and cryopreserved PBMCs, with the exceptions of total CD3 lymphocytes and CD4 memory lymphocytes. Although statistically significant, on examining the actual numerical differences many are unlikely to be of clinical importance. Exceptions include activated CD4 and CD8 subsets, naïve CD4 and memory CD8 T-cells that demonstrate a much larger change from fresh to frozen. Although isotype controls and fluorescence-minus-one gating strategies were applied, inter-operator variation between the two scientists analysing the data at each site may play a role in explaining why such a difference might occur. Although CD4 and CD8 subsets are unlikely to be activated by the cryopreservation-thaw process, this phenomenon could possibly be explained by soluble HLA-DR that attaches during the cryopreservation-thaw process. For activated CD3 and CD4 T-cells, a higher percentage of circulating monocytes and lymphocytes appeared to be associated with less difference between the whole blood assay and cryopreserved PBMCs, in contrast to higher percentage of granulocytes and eosinophils associated with a greater difference. Since this relationship was not apparent with any of the other lymphocyte subsets examined, it is more likely to be an incidental finding than due to preferential loss of a leukocyte class.

Unless appropriate control groups are used applying strict cryopreservation, storage and thawing protocols, caution should be applied in the interpretation of research

studies that investigate immune-reconstitution and functional responses to HIV using frozen PBMCs. These findings are reflective of a smaller study (n=40) which showed that cryopreservation induces a decrease in naïve CD4 T-cells and an increase in effector CD8 T-cells [129].

Exploration of the impact of recent illness on differences between fresh and frozen cells revealed less difference between the two assays. This might be explained by the longevity of the lymphocytes themselves, as younger lymphocytes, or those with less replicative history, may be less sensitive to the cryopreservation and thawing processes. Following an infection, new effector memory CD8 T-cells are likely to be in circulation and the high thymic output in healthy children may compensate for the loss of naïve T-cells as they differentiate into memory cells.

The primary finding when examining the difference between the measures of CD4 T-cells by the whole blood assay and cryopreserved PBMCs in both the CWC and CHER was that the healthy children had 2.9% less and the HIV-infected children had 2.5% more CD4 in the cryopreserved specimens. This may be explained by the storage protocol used for the CWC specimens with initial storage at -80°C for 6 months and thereafter transferred to liquid nitrogen, whereas all CHER PBMCs were stored in liquid nitrogen after gradual cooling in a Nalgene® freezing container over 24 hours. There is good evidence that PBMC specimens can remain viable for up to 12 years with correct cryopreservation and storage[148]. Although literature suggests up to 18 months at -80°C is acceptable for immunophenotyping purposes[149], temperature fluctuation [150] may have affected cell viability. In contrast, the modest increase in the cryopreserved CD4 of the CHER participants could possibly be due to preferential cell death from cryopreservation-thawing of a combination of cell groups (e.g. monocytes, natural killer cells and CD8 T-cells) since analysis performed on individual cell groups did not reveal a single cause.

The other finding of the CHER whole blood assay versus cryopreserved PBMCs analysis was the increased odds of a difference, either an increase or decrease, between fresh whole blood assay and cryopreserved PBMCs if the child had reached a trial-defined endpoint. This might suggest clinical deterioration and/or poor virological control could cause immunological instability that could make CD4 T-cells more vulnerable during cell preparation or the cryopreservation-thaw process.

Due to the wide variability of the difference between the whole blood assay and cryopreserved PBMCs within subsets seen here and also reported elsewhere [128], it is difficult to interpret the significance of the live cell percentage. It is vital that a viability stain is used with cryopreserved PBMCs since dead cells can generate artifacts as a result of nonspecific antibody binding or uptake of fluorescent probes. Establishing a minimum value for the percentage of live cells required to preserve an accurate distribution of lymphocyte subsets is appealing. One study reports a cut-off of 75% live cells to be appropriate [151], however data presented here indicates that no cut-off reliably identifies an optimal viability quotient.

This study compiles data from three separate analyses to piece together the effect of cryopreservation and differences in methodology on immunophenotypes of lymphocyte subsets (fresh versus frozen PBMCs, whole blood assay versus cryopreserved PBMCs in healthy children, and whole blood assay versus cryopreserved PBMCs in HIV-infected children). Although relatively small numbers, the fresh versus frozen PBMC analysis offers the most direct comparison and demonstrates the least differences. This suggests that differences in methodologies are likely to play a more important role. In the whole blood assay versus cryopreserved PBMCs in healthy children a number of factors may have influenced the results: overnight shipping by airfreight [152], processing 24 hours after venipuncture (may reduce PBMC yield [153]), different fluorochromes, flowcytometers and gating-strategies analysed by different individuals, even use of different buffers and media have been reported to influence results [154].

With the exception of activated T-cells, the differences seen between the whole blood assay and cryopreserved PBMCs are unlikely to have significance in clinical practice, however statistical adjustment for these changes in large clinical and epidemiological studies may be a reasonable consideration [155]. No clear pattern for preferential cell death was identified, inferring that either the effect of cryopreservation is entirely non-specific or more complex than explored here. However, where possible PBMC cryopreservation and storage should probably avoid -80°C and use liquid nitrogen for optimal cell viability. The causes of the differences between the whole blood assay and cryopreserved PBMCs are largely likely to be methodological, therefore caution must be applied when comparing studies with different methodologies for immunophenotyping, and also when interpreting studies performed on frozen cells unless appropriate comparison groups are used.

Chapter 4

Immunophenotypic responses to HIV and ART in children

4.1 Background

The deleterious effect of HIV on CD4 T-cells is a defining feature of HIV infection and the critical role of CD8 T-cells in the cellular immune response to HIV is widely recognized; however there is much that is not well understood about the immunology of HIV infection in paediatrics and the child's immune recovery with ART. The use of immunological research is key to develop our understanding of the immune system and its role in combating infections such as HIV.

Primarily we need to appreciate the range of immunological disturbances that exist with HIV infection in children, and whether these changes can be resolved with ART. As ART strategies evolve, it is imperative to describe the immunological response to ART-interruption in the short and long term, and whether these responses off ART are dependent on the age of the child and stage of the developing immune system. Understanding the range of immunophenotypic responses to HIV and ART might

help identify novel treatment modalities or mechanisms of therapeutic monitoring to improve the care of HIV-infected children and optimise their immunological health.

Each measurement of thymic output from the Child Wellness Clinic (CWC) or CHER cohort was complemented by a profile of immunological phenotypes. These profiles have been used to describe some of the immunological responses to HIV and ART by comparing HIV-uninfected unexposed South African children with HIV-infected children from the CHER trial, and within HIV-infected children according to the CHER ART-strategies used. The large numbers of specimens and children examined from both the CWC and the CHER trial present a unique opportunity to further understand the immune dynamics of HIV infection and immune recovery with ART. It is also intended that these findings might help contextualize the outcomes of the thymic output analysis of Chapter Five.

4.2 Results

4.2.1 Immunophenotypes of HIV-uninfected South African children compared with HIV-infected participants from CHER

The immunophenotypes measured by Panels A and B (Table 2.3, section 2.4.2) in both the CWC and ART-96W from the CHER trial have been compared and presented in Table 4.1. Here the age groups are different to the previous chapter because sampling took place around specific CHER trial study visits (i.e. 1-2 months, 2-4 months etc). Initial exploration of the data revealed the immunophenotypic measurements were not normally distributed but skewed towards the left, therefore a log-scaled t-test has been used to analyse these data.

The majority of the significant differences are in the first two age groups: where ART had not been commenced and therefore virological control had not yet been attained (Figure 5.11, Chapter 5), and the immunological benefits of ART are not seen. Compared to the HIV-uninfected healthy controls, children from ART-96W aged 1-4 months had significantly higher total, naïve, memory, overall proliferation and activation of cytotoxic T-cells; higher total, naive and proliferation of B-cells; higher total and proliferation of natural killer cells; and lower CD4/CD8 ratio. From 4 months to 2 years, the benefits of continuous ART are demonstrated by ART-96W since many of the lymphocyte subsets became similar to the HIV-uninfected controls.

However when ART was interrupted at 2 years, immunophenotypic differences returned in the 2-4 year age group, despite 35% of the samples examined being on ART, with higher naïve CD4 T-cells and naïve CD4 T-cell proliferation; higher total, naïve, naïve-proliferation and activation of CD8 T-cells; and lower CD4/CD8 ratio than the HIV-uninfected controls. In the final age group (4-8 years) 49% of ART-96W had resumed ART and the overall group had higher total T-cells, higher naïve CD4 T-cells, continued higher levels of total, naïve, overall proliferation and activation of CD8 T-cells; and continued lower CD4/CD8 ratio than the controls. Further analysis of the 2-4 year age group from ART-96W revealed no significant differences in each lymphocyte subset between those on or off ART (data not shown, all p-values >0.1), likely due to a plausible delay in achieving virological control after re-commencing ART. At 4-8 years a few significant differences were seen with children from ART-96W off ART having higher proliferation of both naïve and memory CD8 T-cells, and

higher activated and memory CD8 T-cells, and lower proliferation of natural killer cells (data not shown, all p-values <0.05) than children from ART-96W on ART. However, when comparing children on ART from ART-96W aged 4-8 years (n=24, median 144 [IQR 132-244] weeks since recommencing ART) with healthy HIV-uninfected children from the CWC at the same age (n=61), a number of significant differences are apparent: higher naïve CD4, naïve CD8*, CD8* and activated CD8 T-cells, and lower CD4/CD8 ratio*, memory CD4*, proliferating naïve CD8, B-cells and naïve B-cells, and total CD3 T-cells (data not shown, all p-values <0.05, *indicates p-value <0.0001).

The main exception to the above-described pattern of immunological dynamics in response to ART is the significantly lower memory CD4 T-cells seen continuously in ART-96W from 1 month to 8 years of age regardless of ART-status.

Table 4.1: Comparison of immunophenotypes of healthy South African children and HIV-infected children. There were 411 healthy HIV-uninfected children from the CWC and 331 samples from 107 HIV-infected children from ART-96W who started on ART at enrolment of CHER (median 7 weeks old), interrupted ART after 96 weeks (~2 years old) and restarted ART after median 70 weeks interruption. In ART-96W no more than one sample per child was included in each age group. Lymphocyte subsets are represented in cells per microlitre of blood. Immunophenotypes were obtained from cryopreserved PBMCs in both cohorts. Welch's t-test compares log-transformed values from each lymphocyte subset within each age group; *highlighted in red indicates p-value statistical significance <0.05. Within the available samples measured, the percentage of children on ART is represented in the header of the table.

TABLE 4.1: Cell group (cells/µl) Median [IQR] % ART-96W on ART		Age ranges 1-2 months (n=28 vs 33) 0%	2-4 months (n=46 vs 41) 73%	4-8 months (n=66 vs 37) 100%	8-12 months (n=40 vs 37) 100%	1-1.5yrs (n=42 vs 35) 100%	1.5-2yrs (n=52 vs 62) 100%	2-4yrs (n=76 vs 37) 35%	4-8yrs (n=61 vs 49) 49%
CD3+	CWC	3728 [2640-5290]	3886 [2488-4454]	4130 [2676-5980]	4133 [2736-5171]	3828 [2735-6002]	3829 [2961-5281]	2869 [2098-3878]	2120 [1548-2816]
	ART-96W	4088 [3044-6527]	4762 [2627-6605]	4315 [3116-5940]	4348 [3244-5723]	3718 [2417-5059]	3296 [2523-4277]	3006 [2066-3746]	2308 [1796-2881]
p-value		0.31	0.23	0.36	0.58	0.18	0.21	0.31	0.007*
CD4+	CWC	2472 [1843-3630]	2597 [1683-3294]	2381 [1353-3939]	2188 [1372-2943]	1969 [1245-3474]	2021 [1297-2901]	1421 [962-2202]	1132 [712-1562]
	ART-96W	2355 [1565-3846]	2804 [1352-4099]	2647 [1352-4099]	2245 [1372-3525]	2038 [1296-3121]	1835 [1250-2519]	1368 [814-1784]	943 [666-1365]
p-value		0.42	0.59	0.18	0.95	0.85	0.73	0.40	0.95
CD4+ CD45RA+	CWC	1805 [858-2975]	1920 [1019-2688]	1758 [734-3248]	1569 [575-2347]	1365 [642-2745]	1378 [748-2171]	742 [314-1501]	558 [204-1012]
	ART-96W	1985 [1207-3434]	2285 [1015-3566]	2176 [1351-3438]	1774 [995-3049]	1618 [974-2586]	1350 [856-1957]	986 [538-1372]	635 [398-1018]
p-value		0.31	0.41	0.0008*	0.13	0.11	0.14	0.02*	0.0002*
CD4+ CD45RA+ KI67+	CWC	59 [22-131]	44 [15-103]	42 [12-114]	26 [6-62]	27 [8-84]	31 [10-90]	22 [7-73]	21 [6-58]
	ART-96W	69 [28-164]	66 [14-170]	27 [14-77]	36 [11-109]	24 [11-48]	23 [10-44]	13 [6-26]	13 [6-38]
p-value		0.20	0.09	0.51	0.07	0.65	0.33	0.008*	0.97
CD4+ CD45RO+	CWC	668 [333-1940]	676 [310-1299]	623 [237-1803]	619 [278-1582]	604 [261-1681]	644 [326-1227]	679 [306-1483]	574 [251-1116]
	ART-96W	370 [167-881]	519 [176-1021]	471 [236-792]	472 [185-969]	420 [222-774]	485 [279-794]	382 [188-605]	307 [169-550]
p-value		<0.0001*	0.0004*	0.0005*	0.006*	<0.0001*	0.003*	<0.0001*	<0.0001*
CD4+ CD45RO+ KI67+	CWC	83 [28-375]	78 [25-216]	73 [21-305]	67 [19-264]	73 [20-276]	77 [27-191]	71 [24-195]	54 [16-138]
	ART-96W	91 [31-254]	95 [24-228]	76 [28-163]	80 [22-237]	62 [25-131]	71 [31-150]	45 [17-99]	41 [18-87]
p-value		0.38	0.63	0.39	0.43	0.12	0.51	0.11	0.24

TABLE 4.1: Cell group (cells/µl) Median [IQR] % ART-96W on ART		Age ranges							
		1-2 months (n=28 vs 33) 0%	2-4 months (n=46 vs 41) 73%	4-8 months (n=66 vs 37) 100%	8-12 months (n=40 vs 37) 100%	1-1.5yrs (n=42 vs 35) 100%	1.5-2yrs (n=52 vs 62) 100%	2-4yrs (n=76 vs 37) 35%	4-8yrs (n=61 vs 49) 49%
CD8+	CWC	892 [648-1521]	923 [529-1274]	1460 [805-2497]	1733 [1004-2297]	1667 [962-2926]	1656 [1045-2483]	1093 [695-1640]	735 [494-1042]
	ART-96W	1583 [1040-3105]	1708 [986-2613]	1565 [979-2249]	1723 [1158-3002]	1562 [931-2240]	1468 [1051-2127]	1666 [1051-2159]	1363 [969-1827]
p-value		<0.0001*	<0.0001*	0.16	0.15	0.21	0.88	<0.0001*	<0.0001*
CD8+ CD45RA+	CWC	569 [337-1192]	590 [230-983]	942 [332-1888]	1035 [330-1798]	1147 [405-2276]	1128 [507-1947]	638 [195-1247]	345 [117-806]
	ART-96W	1029 [554-2285]	1180 [513-2140]	1225 [638-1860]	1261 [721-2314]	1245 [692-1919]	1150 [760-1795]	1217 [643-1718]	934 [576-1440]
p-value		0.0003*	<0.0001*	0.0001*	0.003	0.32	0.008*	<0.0001*	<0.0001*
CD8+ CD45RA+ KI67+	CWC	31 [12-95]	32 [8-86]	54 [13-170]	57 [11-159]	60 [16-160]	51 [16-118]	31 [6-79]	21 [5-63]
	ART-96W	106 [40-384]	92 [22-225]	57 [27-127]	76 [35-243]	57 [22-121]	58 [29-124]	52 [21-107]	51 [22-109]
p-value		<0.0001*	<0.0001*	0.02*	0.008	0.89	0.92	<0.0001*	<0.0001*
CD8+ CD45RO+	CWC	322 [140-730]	333 [121-721]	518 [196-1468]	697 [218-1542]	520 [214-1694]	527 [225-1277]	455 [166-1179]	390 [112-794]
	ART-96W	554 [275-1450]	528 [179-1254]	338 [169-783]	462 [265-1132]	317 [134-576]	318 [164-606]	448 [221-879]	429 [205-742]
p-value		0.006*	0.05	0.06	0.42	0.0008*	0.02*	0.49	0.06
CD8+ CD45RO+ KI67+	CWC	45 [13-157]	44 [10-206]	88 [21-429]	109 [19-435]	89 [25-405]	73 [19-276]	51 [12-183]	37 [7-136]
	ART-96W	289 [109-967]	170 [30-508]	75 [26-220]	101 [45-319]	61 [18-148]	64 [27-150]	64 [22-185]	61 [23-148]
p-value		<0.0001*	0.005*	0.65	0.43	0.01*	0.004*	0.05	0.002*
CD4+ HLADR+	CWC	101 [59-236]	125 [57-221]	109 [38-265]	100 [41-231]	103 [45-283]	140 [60-248]	142 [70-353]	134 [56-289]
	ART-96W	114 [57-295]	145 [47-265]	124 [59-250]	154 [64-343]	124 [63-268]	144 [74-252]	117 [74-191]	87 [42-192]
p-value		0.53	0.68	0.28	0.16	0.18	0.52	0.07	0.12

TABLE 4.1: Cell group (cells/µl) Median [IQR] % ART-96W on ART		Age ranges 1-2 months (n=28 vs 33) 0%	2-4 months (n=46 vs 41) 73%	4-8 months (n=66 vs 37) 100%	8-12 months (n=40 vs 37) 100%	1-1.5yrs (n=42 vs 35) 100%	1.5-2yrs (n=52 vs 62) 100%	2-4yrs (n=76 vs 37) 35%	4-8yrs (n=61 vs 49) 49%
CD8+ HLADR+	CWC	72 [33-219]	81 [29-336]	241 [77-921]	343 [138-779]	320 [119-907]	489 [132-1057]	380 [143-859]	283 [151-547]
	ART-96W	737 [323-2111]	526 [144-1450]	318 [105-1014]	619 [226-2014]	606 [261-1145]	638 [344-1135]	991 [511-1509]	780 [417-1361]
p-value		<0.0001*	<0.0001*	0.06	0.009*	0.01*	0.003*	<0.0001*	<0.0001*
CD19+	CWC	842 [492-1704]	954 [427-1359]	1174 [531-2088]	1246 [669-1840]	1015 [563-1908]	1113 [706-1989]	709 [363-1097]	400 [220-751]
	ART-96W	1473 [793-2377]	1711 [973-2846]	1423 [950-2846]	1455 [682-2132]	1434 [907-1959]	937 [582-1402]	418 [292-501]	345 [225-501]
p-value		0.005*	0.0002*	0.001*	0.33	0.10	0.12	0.85	0.36
CD19+ KI67+	CWC	99 [52-248]	62 [24-145]	70 [26-171]	63 [29-141]	50 [21-137]	59 [27-162]	45 [18-93]	28 [11-66]
	ART-96W	198 [77-387]	164 [58-369]	73 [40-120]	74 [28-157]	59 [31-129]	48 [20-98]	36 [23-47]	21 [11-39]
p-value		0.005*	<0.0001*	0.11	0.22	0.33	0.01*	0.63	0.19
CD19+ IGD+	CWC	733 [412-1537]	837 [361-1228]	1038 [456-1879]	1076 [566-1636]	864 [459-1657]	872 [542-1661]	504 [238-862]	269 [138-562]
CD27-	ART-96W	1307 [675-2170]	1509 [832-2623]	1274 [830-2280]	1244 [522-1909]	1207 [728-1733]	775 [447-1198]	310 [200-399]	244 [140-375]
p-value		0.18	0.02*	0.05	0.53	0.13	0.15	0.56	0.38
CD19+ IGD+	CWC	77 [37-194]	50 [20-113]	43 [14-92]	46 [15-99]	28 [11-71]	29 [13-71]	21 [7-50]	12 [4-33]
CD27- KI67+	ART-96W	131 [54-319]	120 [45-325]	55 [30-140]	51 [16-105]	42 [19-77]	27 [12-57]	17 [9-24]	9 [4-16]
p-value		0.16	0.003*	0.03*	0.38	0.07	0.53	0.71	0.09
CD56+	CWC	417 [222-891]	292 [153-533]	347 [179-685]	327 [180-573]	301 [173-627]	336 [247-607]	276 [152-524]	177 [92-355]
	ART-96W	467 [286-981]	526 [273-991]	369 [226-703]	300 [190-589]	283 [140-714]	248 [155-398]	430 [286-539]	153 [75-280]
p-value		0.24	0.003*	0.27	0.80	0.76	0.003*	0.24	0.26

TABLE 4.1: Cell group (cells/µl) Median [IQR] % ART-96W on ART		Age ranges							
		1-2 months (n=28 vs 33) 0%	**2-4 months** (n=46 vs 41) 73%	**4-8 months** (n=66 vs 37) 100%	**8-12 months** (n=40 vs 37) 100%	**1-1.5yrs** (n=42 vs 35) 100%	**1.5-2yrs** (n=52 vs 62) 100%	**2-4yrs** (n=76 vs 37) 35%	**4-8yrs** (n=61 vs 49) 49%
CD56+ KI67+	CWC	80 [33-196]	43 [15-113]	47 [16-117]	42 [17-94]	39 [17-107]	45 [23-103]	35 [14-96]	23 [8-63]
	ART-96W	114 [54-315]	141 [45-318]	69 [27-191]	53 [28-146]	44 [18-153]	38 [18-85]	113 [64-163]	21 [8-50]
p-value		0.007*	<0.0001*	0.0006*	0.01*	0.36	0.29	0.26	0.64
CD4/CD8 Ratio	CWC	2.9 [1.9-3.3]	2.6 [2.1-4.0]	1.8 [1.1-2.5]	1.4 [1.0-1.9]	1.1 [0.8-1.7]	1.2 [0.8-1.6]	1.4 [1.0-2.0]	1.5 [1.1-2.0]
	ART-96W	1.4 [0.9-2.1]	1.4 [1.1-2.2]	1.7 [1.4-2.0]	1.1 [0.7-1.9]	1.3 [1.0-2.0]	1.2 [0.9-1.5]	0.9 [0.6-1.0]	0.7 [0.5-0.9]
p-value		<0.0001*	<0.0001*	0.83	0.16	0.28	0.86	<0.0001*	<0.0001*

4.2.2 Impact of CHER ART-strategies on immunophenotypes

A) Early versus deferred ART

Comparison of early versus deferred ART is detailed in Table 4.2 comparing all available data from ART-Def (n=200 samples) and ART-96W (n=311 samples) from enrolment until 8 years. The data demonstrates a number of differences between 2 to 8 months, but thereafter the parameters are quite comparable. The most significant changes are higher CD3, total, naïve CD4, naïve and memory CD8, and lower memory and memory-proliferation of CD8 and CD4/CD8 ratio in the 4-8 month age group. It is important to note that 79% of children aged 8-12 months in ART-Def had commenced ART, and 100% of children by 1-1.5 years.

Table 4.2: Comparison of immunophenotypes of ART-Def and ART-96W. HIV-infected children who initiated ART at clinical/immunological progression then continued on ART (ART-Def, n=200 samples from 82 children) compared with children started on ART at enrolment of the CHER trial (median 7 weeks of age), ART-interrupted after 96 weeks (~2 years of age) and restarted ART after median 70 weeks interruption (ART-96W, n=331 samples from 107 children). No more than one sample per child was included in each age group. Lymphocyte subsets are represented in cells per microlitre of blood. Immunophenotypes were obtained from cryopreserved PBMCs in both cohorts. Welch's t-test compares log-transformed values from each lymphocyte subset within each age group; *highlighted in red indicates p-value statistical significance <0.05. Within the available samples measured, the percentage of children on ART is represented in the header of the table.

TABLE 4.2: Cell group (cells/µl) Median: [IQR]: % ART-Def on ART: % ART-96W on ART:		Age ranges							
		1-2 months (n=24 vs 33) 0% 0%	2-4 months (n=20 vs 41) 0% 73%	4-8 months (n=19 vs 37) 58% 100%	8-12 months (n=47 vs 37) 79% 100%	1-1.5yrs (n=16 vs 35) 100% 100%	1.5-2yrs (n=25 vs 62) 88% 100%	2-4yrs (n=36 vs 37) 97% 35%	4-8yrs (n=13 vs 49) 100% 49%
CD3+	ART-Def	5208 [3087-6456]	3271 [2357-4530]	2890 [1865-4398]	3497 [2606-4839]	3669 [2553-3920]	3697 [3111-5197]	3323 [2431-3920]	2207 [1939-2873]
	ART-96W	4088 [3044-6527]	4762 [2627-6605]	4315 [3116-5940]	4348 [3244-5723]	3718 [2417-5059]	3296 [2523-4277]	3006 [2066-3746]	2308 [1796-2881]
p-value		0.76	0.09	0.009*	0.14	0.37	0.15	0.73	0.83
CD4+	ART-Def	2707 [1146-3585]	1554 [1057-2474]	1079 [579-1757]	1674 [1270-2366]	1642 [1364-2157]	1966 [1371-2168]	1496 [1151-1973]	1150 [880-1309]
	ART-96W	2355 [1565-3846]	2804 [1352-4099]	2647 [1352-4099]	2245 [1372-3525]	2038 [1296-3121]	1835 [1250-2519]	1368 [814-1784]	943 [666-1365]
p-value		0.74	0.02*	<0.0001*	0.23	0.09	0.59	0.21	0.29
CD4+ CD45RA+	ART-Def	2250 [862-3002]	1222 [832-2050]	834 [386-1392]	1255 [963-1954]	1293 [1031-1650]	1470 [945-1662]	1059 [740-1552]	772 [526-982]
	ART-96W	1985 [1207-3434]	2285 [1015-3566]	2176 [1351-3438]	1774 [995-3049]	1618 [974-2586]	1350 [856-1957]	986 [538-1372]	635 [398-1018]
p-value		0.62	0.03*	<0.0001*	0.31	0.05	0.58	0.25	0.28
CD4+ CD45RA+ KI67+	ART-Def	57 [39-79]	51 [27-64]	20 [13-35]	23 [18-32]	18 [15-19]	19 [12-30]	15 [10-26]	10 [6-21]
	ART-96W	69 [28-164]	66 [14-170]	27 [14-77]	36 [11-109]	24 [11-48]	23 [10-44]	13 [6-26]	13 [6-38]
p-value		0.67	0.24	0.09	0.17	0.03*	0.49	0.24	0.14
CD4+ CD45RO+	ART-Def	425 [304-562]	310 [199-366]	237 [136-421]	397 [287-497]	356 [288-411]	462 [352-531]	417 [304-494]	342 [288-405]
	ART-96W	370 [167-881]	519 [176-1021]	471 [236-792]	472 [185-969]	420 [222-774]	485 [279-794]	382 [188-605]	307 [169-550]
p-value		0.48	0.03*	0.01*	0.28	0.08	0.95	0.36	0.30
CD4+ CD45RO+ KI67+	ART-Def	115 [82-165]	77 [44-91]	68 [34-79]	70 [52-94]	43 [37-64]	49 [43-74]	47 [34-72]	29 [20-48]
	ART-96W	91 [31-254]	95 [24-228]	76 [28-163]	80 [22-237]	62 [25-131]	71 [31-150]	45 [17-99]	41 [18-87]
p-value		0.21	0.22	0.19	0.78	0.08	0.11	0.68	0.22

TABLE 4.2: Cell group (cells/µl) Median: [IQR]: % ART-Def on ART: % ART-96W on ART:		Age ranges							
		1-2 months (n=24 vs 33) 0% 0%	2-4 months (n=20 vs 41) 0% 73%	4-8 months (n=19 vs 37) 58% 100%	8-12 months (n=47 vs 37) 79% 100%	1-1.5yrs (n=16 vs 35) 100% 100%	1.5-2yrs (n=25 vs 62) 88% 100%	2-4yrs (n=36 vs 37) 97% 35%	4-8yrs (n=13 vs 49) 100% 49%
CD8+	ART-Def	2431 [1527-3021]	1341 [724-2005]	1720 [1053-2473]	1632 [1261-2373]	1661 [1239-2206]	1432 [1159-2486]	1666 [1019-2304]	1131 [1011-1610]
	ART-96W	1583 [1040-3105]	1708 [986-2613]	1565 [979-2249]	1723 [1158-3002]	1562 [931-2240]	1468 [1051-2127]	1666 [1051-2159]	1363 [969-1827]
p-value		0.36	0.34	0.74	0.35	0.66	0.16	0.62	0.66
CD8+ CD45RA+	ART-Def	1175 [587-1649]	640 [408-1070]	843 [498-1117]	1255 [595-1538]	1229 [880-1615]	1111 [921-1849]	1178 [735-1502]	866 [744-1192]
	ART-96W	1029 [554-2285]	1180 [513-2140]	1225 [638-1860]	1261 [721-2314]	1245 [692-1919]	1150 [760-1795]	1217 [643-1718]	934 [576-1440]
p-value		0.73	0.05	0.02*	0.19	0.66	0.18	0.89	0.82
CD8+ CD45RA+ KI67+	ART-Def	136 [75-254]	48 [39-113]	77 [43-120]	66 [47-105]	36 [33-61]	51 [34-77]	43 [26-79]	24 [19-40]
	ART-96W	106 [40-384]	92 [22-225]	57 [27-127]	76 [35-243]	57 [22-121]	58 [29-124]	52 [21-107]	51 [22-109]
p-value		0.83	0.81	0.63	0.38	0.62	0.44	0.68	0.07
CD8+ CD45RO+	ART-Def	991 [529-1501]	622 [286-1076]	816 [395-1288]	522 [302-737]	331 [246-444]	400 [271-636]	382 [210-530]	330 [244-493]
	ART-96W	554 [275-1450]	528 [179-1254]	338 [169-783]	462 [265-1132]	317 [134-576]	318 [164-606]	448 [221-879]	429 [205-742]
p-value		0.04*	0.10	0.01*	0.98	0.45	0.05	0.22	0.50
CD8+ CD45RO+ KI67+	ART-Def	609 [281-809]	195 [130-567]	269 [98-364]	114 [77-205]	48 [33-83]	54 [34-76]	52 [26-104]	36 [17-42]
	ART-96W	289 [109-967]	170 [30-508]	75 [26-220]	101 [45-319]	61 [18-148]	64 [27-150]	64 [22-185]	61 [23-148]
p-value		0.03*	0.32	0.002*	0.72	0.84	0.72	0.38	0.28
CD4+ HLADR+	ART-Def	153 [109-166]	137 [58-166]	113 [66-178]	149 [103-211]	123 [95-155]	147 [114-207]	119 [85-162]	96 [92-111]
	ART-96W	114 [57-295]	145 [47-265]	124 [59-250]	154 [64-343]	124 [63-268]	144 [74-252]	117 [74-191]	87 [42-192]
p-value		0.51	0.32	0.41	0.32	0.45	0.28	0.92	0.53

TABLE 4.2: Cell group (cells/µl) Median: [IQR]: % ART-Def on ART: % ART-96W on ART:		Age ranges							
		1-2 months (n=24 vs 33) 0% 0%	2-4 months (n=20 vs 41) 0% 73%	4-8 months (n=19 vs 37) 58% 100%	8-12 months (n=47 vs 37) 79% 100%	1-1.5yrs (n=16 vs 35) 100% 100%	1.5-2yrs (n=25 vs 62) 88% 100%	2-4yrs (n=36 vs 37) 97% 35%	4-8yrs (n=13 vs 49) 100% 49%
CD8+ HLADR+	ART-Def	1124 [541-1926]	827 [323-1336]	1034 [445-1967]	855 [524-1205]	754 [227-1250]	649 [504-1302]	731 [349-1208]	545 [465-670]
	ART-96W	737 [323-2111]	526 [144-1450]	318 [105-1014]	619 [226-2014]	606 [261-1145]	638 [344-1135]	991 [511-1509]	780 [417-1361]
p-value		0.18	0.40	0.02*	0.17	0.88	0.07	0.11	0.18
CD19+	ART-Def	1625 [1080-2300]	1280 [703-2038]	900 [751-1772]	1206 [990-1792]	1672 [1300-2085]	763 [656-1037]	847 [432-1112]	372 [233-521]
	ART-96W	1473 [793-2377]	1711 [973-2846]	1423 [950-2846]	1455 [682-2132]	1434 [907-1959]	937 [582-1402]	418 [292-501]	345 [225-501]
p-value		0.40	0.17	0.15	0.88	0.47	0.47	0.32	0.76
CD19+ KI67+	ART-Def	224 [140-299]	141 [63-202]	70 [48-93]	64 [50-91]	77 [55-107]	31 [26-47]	44 [26-51]	17 [10-29]
	ART-96W	198 [77-387]	164 [58-369]	73 [40-120]	74 [28-157]	59 [31-129]	48 [20-98]	36 [23-47]	21 [11-39]
p-value		0.41	0.55	0.33	0.72	0.52	0.04*	0.54	0.26
CD19+ IGD+	ART-Def	1368 [920-2042]	1128 [636-1871]	724 [605-1589]	1026 [827-1499]	1507 [1202-1857]	606 [494-900]	683 [298-904]	224 [146-398]
CD27-	ART-96W	1307 [675-2170]	1509 [832-2623]	1274 [830-2280]	1244 [522-1909]	1207 [728-1733]	775 [447-1198]	310 [200-399]	244 [140-375]
p-value		0.43	0.20	0.13	0.60	0.39	0.39	0.32	0.96
CD19+ IGD+	ART-Def	140 [93-258]	102 [45-179]	35 [25-39]	42 [26-53]	65 [44-77]	22 [13-26]	20 [14-30]	5 [4-7]
CD27- KI67+	ART-96W	131 [54-319]	120 [45-325]	55 [30-140]	51 [16-105]	42 [19-77]	27 [12-57]	17 [9-24]	9 [4-16]
p-value		0.55	0.42	0.10	0.99	0.54	0.06	0.70	0.09

TABLE 4.2: Cell group (cells/µl) Median: [IQR]: % ART-Def on ART: % ART-96W on ART:		Age ranges							
		1-2 months (n=24 vs 33) 0% 0%	2-4 months (n=20 vs 41) 0% 73%	4-8 months (n=19 vs 37) 58% 100%	8-12 months (n=47 vs 37) 79% 100%	1-1.5yrs (n=16 vs 35) 100% 100%	1.5-2yrs (n=25 vs 62) 88% 100%	2-4yrs (n=36 vs 37) 97% 35%	4-8yrs (n=13 vs 49) 100% 49%
CD56+	ART-Def	571 [352-858]	301 [222-536]	200 [157-302]	313 [210-479]	197 [159-273]	215 [148-416]	256 [138-344]	157 [97-301]
	ART-96W	467 [286-981]	526 [273-991]	369 [226-703]	300 [190-589]	283 [140-714]	248 [155-398]	430 [286-539]	153 [75-280]
p-value		0.69	0.02*	0.05	0.90	0.11	0.81	0.18	0.90
CD56+ KI67+	ART-Def	174 [76-244]	93 [55-116]	39 [31-60]	57 [31-76]	33 [30-44]	30 [21-47]	40 [22-46]	16 [12-30]
	ART-96W	114 [54-315]	141 [45-318]	69 [27-191]	53 [28-146]	44 [18-153]	38 [18-85]	113 [64-163]	21 [8-50]
p-value		0.53	0.03*	0.21	0.64	0.13	0.33	0.26	0.63
CD4/CD8 Ratio	ART-Def	1.2 [0.5-1.7]	1.4 [0.7-2.4]	0.8 [0.4-1.2]	1.0 [0.8-1.4]	1.0 [0.7-1.5]	1.0 [0.8-1.5]	1.0 [0.6-1.4]	0.9 [0.8-1.2]
	ART-96W	1.4 [0.9-2.1]	1.4 [1.1-2.2]	1.7 [1.4-2.0]	1.1 [0.7-1.9]	1.3 [1.0-2.0]	1.2 [0.9-1.5]	0.9 [0.6-1.0]	0.7 [0.5-0.9]
p-value		0.26	0.28	0.0002*	0.71	0.06	0.40	0.06	0.10

B) ART-interruption after 40 or 96 weeks ART

The effect of ART-interruption after 40 weeks of ART was shown by comparing immunophenotypes of ART-40W with ART-96W at 60 weeks which was when ART-40W interrupted for a median of 20 [IQR: 18.5-20] weeks (since 26% had restarted ART by 60 weeks, n=27) and ART-96W would still be on ART (n=35)(Table 4.3). ART-40W at 60 weeks had significantly lower CD4/CD8 ratio with lower CD4, naïve and memory CD4. At 60 weeks ART-40W also had significantly higher memory CD8 with higher CD8 memory proliferation and higher CD8 activation, and lower overall B-cells and naïve B-cells.

At 96 weeks 29/47 (62%) children examined from ART-40W had recommenced ART. Many of the differences seen at 60 weeks had resolved with the exceptions of on-going significantly higher memory CD8 and activated CD8. The CD4/CD8 ratio also remained lower in ART-40W but to a less significant extent. Subsequently at 152 and 248 trial weeks none of the immune parameters compared were significant.

Table 4.3: Comparison of immunophenotypes of ART-40W and ART-96W. ART-40W (n=129 samples in 68 children) compared to ART-96W (n=134 samples in 70 children), compares the impact on immunophenotypic profiles of ART-interruption after 40 or 96 weeks of ART, at approximately one or two years of age respectively. In both groups ART was initiated at age 7.3 weeks (IQR: 6.4-9.0). Median duration of ART-interruption for ART-40W was 33 (26-45) and 70 (35-109) weeks in ART-96W. Lymphocyte subsets are represented in cells per mircolitre of blood. Immunophenotypes were obtained from cryopreserved PBMCs. Welch's t-test compares log-transformed values from each lymphocyte subset within each age group; *highlighted in red indicates p-value statistical significance <0.05. Within the available samples measured, the percentage of children on ART is represented in the header of the table. NB. B-cell markers were not performed at week 152.

TABLE 4.3:		**Age ranges / CHER trial weeks**			
Cell group (cells/µl) Median: [IQR]:		**Week 60** **1.3 yrs** (n=27 vs 35)	**Week 96** **2 yrs** (n=47 vs 44)	**Week 152** **3 yrs** (n=9 vs 17)	**Week 248** **5 yrs** (n=41 vs 36)
% on ART in ART-40W: % on ART in ART-96W:		26% 100%	62% 100%	89% 35%	66% 50%
CD3+	ART-40W	3069 [2492-4014]	3058 [2211-4160]	2670 [2323-3305]	2152 [1803-2783]
	ART-96W	3849 [2606-4770]	3081 [2629-4000]	3141 [2134-3625]	2350 [1953-2723]
p-value		0.21	0.65	0.77	0.56
CD4+	ART-40W	1206 [1005-1780]	1351 [985-1798]	1249 [1017-1505]	1018 [683-1335]
	ART-96W	1897 [1207-2766]	1808 [1137-2129]	1190 [870-1802]	1029 [704-1307]
p-value		0.002*	0.06	0.90	0.85
CD4+ CD45RA+	ART-40W	977 [746-1360]	1005 [705-1254]	908 [736-1092]	647 [424-914]
	ART-96W	1463 [859-2277]	1256 [804-1598]	842 [625-1324]	663 [432-887]
p-value		0.004*	0.06	0.86	0.99
CD4+ CD45RA+ KI67+	ART-40W	15 [13-26]	16 [11-21]	10 [5-22]	15 [8-28]
	ART-96W	22 [15-34]	21 [16-30]	11 [8-19]	13 [9-25]
p-value		0.05	0.03	0.65	0.91
CD4+ CD45RO+	ART-40W	285 [205-424]	371 [296-518]	309 [281-500]	340 [216-431]
	ART-96W	440 [282-582]	400 [351-530]	376 [255-477]	325 [248-378]
p-value		0.002*	0.17	0.90	0.79
CD4+ CD45RO+ KI67+	ART-40W	58 [36-73]	56 [35-83]	34 [28-46]	41 [30-57]
	ART-96W	56 [47-83]	61 [41-80]	40 [33-57]	46 [31-57]
p-value		0.18	0.18	0.75	0.94
CD8+	ART-40W	1566 [1154-2418]	1555 [1152-2292]	1381 [1080-1709]	1297 [927-1626]
	ART-96W	1863 [1041-2308]	1609 [1098=1963]	1503 [1099-1988]	1357 [1071-1714]
p-value		0.46	0.33	0.77	0.31
CD8+ CD45RA+	ART-40W	1046 [742-1378]	1021 [745-1661]	1006 [795-1272]	843 [652-1249]
	ART-96W	1234 [825-1712]	1231 [824-1479]	1056 [668-1531]	868 [702-1178]
p-value		0.50	0.90	0.96	0.58
CD8+ CD45RA+ KI67+	ART-40W	69 [46-97]	65 [38-114]	40 [27-62]	54 [36-72]
	ART-96W	56 [31-105]	59 [36-85]	39 [24-87]	53 [36-85]
p-value		0.16	0.48	0.99	0.62
CD8+ CD45RO+	ART-40W	449 [294-903]	380 [287-685]	372 [309-438]	324 [253-424]
	ART-96W	305 [179-509]	296 [186-513]	348 [271-854]	399 [286-560]
p-value		0.01*	0.03*	0.62	0.10

TABLE 4.3:		**Age ranges / CHER trial weeks**			
Cell group (cells/µl) Median: [IQR]: % on ART in ART-40W: % on ART in ART-96W		**Week 60** **1.3 yrs** (n=27 vs 35) 26% 100%	**Week 96** **2 yrs** (n=47 vs 44) 62% 100%	**Week 152** **3 yrs** (n=9 vs 17) 89% 35%	**Week 248** **5 yrs** (n=41 vs 36) 66% 50%
CD8+ CD45RO+ KI67+	ART-40W	195 [79-251]	69 [38-138]	56 [23-86]	50 [28-81]
	ART-96W	58 [32-128]	52 [37-98]	57 [38-120]	73 [32-99]
p-value		0.0001*	0.31	0.47	0.12
CD4+ HLADR+	ART-40W	106 [82-142]	139 [94-192]	119 [84-139]	98 [69-135]
	ART-96W	130 [107-160]	127 [102-156]	114 [82-140]	86 [64-119]
p-value		0.12	0.89	0.76	0.56
CD8+ HLADR+	ART-40W	1107 [573-1853]	885 [517-1463]	802 [707-1044]	710 [461-967]
	ART-96W	543 [330-1231]	594 [405-817]	972 [545-1694]	721 [586-1059]
p-value		0.005*	0.002*	0.56	0.25
CD19+	ART-40W	903 [562-1185]	735 [548-984]	NA	300 [212-408]
	ART-96W	1274 [945-1833]	838 [539-1212]	NA	361 [287-443]
p-value		0.003*	0.31		0.29
CD19+ KI67+	ART-40W	50 [35-69]	38 [28-68]	NA	21 [16-30]
	ART-96W	62 [39-80]	41 [33-59]	NA	25 [17-32]
p-value		0.27	0.90		0.47
CD19+ IGD+ CD27-	ART-40W	658 [430-1020]	565 [369-701]	NA	195 [128-276]
	ART-96W	1046 [775-1498]	650 [409-991]	NA	240 [196-329]
p-value		0.002*	0.08		0.23
CD19+ IGD+ CD27- KI67+ p-value	ART-40W	28 [20-43]	21 [13-34]	NA	7 [5-12]
	ART-96W	40 [27-58]	28 [17-37]	NA	9 [7-15]
		0.17	0.18		0.44
CD56+	ART-40W	258 [172-329]	207 [148-357]	NA	162 [119-271]
	ART-96W	270 [192-379]	251 [157-385]	NA	151 [102-280]
p-value		0.07	0.82		0.97
CD56+ KI67+	ART-40W	41 [25-65]	32 [22-72]	NA	24 [16-39]
	ART-96W	41 [29-68]	35 [26-54]	NA	24 [15-38]
p-value		0.16	0.87		0.85
CD4/CD8 Ratio	ART-40W	0.72 [0.45-1.19]	0.89 [0.69-1.22]	0.91 [0.78-0.96]	0.81 [0.6-1.08]
	ART-96W	1.29 [0.97-1.82]	1.21 [0.77-1.44]	0.95 [0.71-1.13]	0.69 [0.5-0.92]
p-value		0.0004*	0.01*	0.78	0.25

C) CHER Long-term perspective

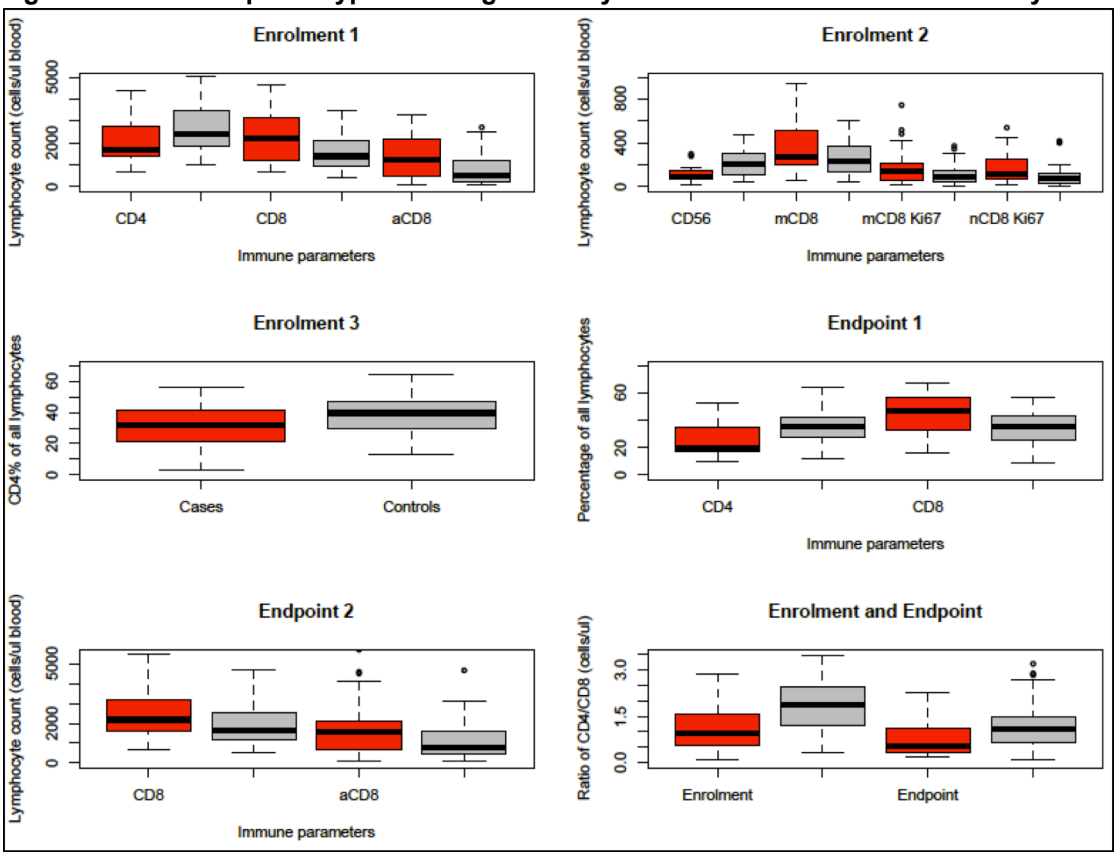
The immunophenotypic profiles of the 3 arms of CHER (ART-Def, ART-40W and ART-96W) were compared at 7-8 years of age and very few differences were revealed (data not shown). Naïve CD4 T-cells were highest (p=0.06) and naïve CD4 proliferation was lowest (p=0.006) in ART-Def, those who had received the longest continuous ART. In contrast, total CD8 T-cells (p=0.01) and naïve CD8 proliferation (p=0.07) were highest in ART-96W who received the least time on ART overall. CD4/CD8 ratio was also lowest in ART-96W (p=0.07). Compared to ART-Def and ART-40W, ART-96W received the least ART overall due to its CHER ART-strategy: early ART for 96 weeks followed by ART interruption until clinical or immunological deterioration. This strategy appeared to allow a longer period off ART compared to ART-40W, and a shorter time on ART compared to ART-Def [68].

4.2.3 Immunophenotypic predictors of ART failure

Whether immunophenotypes at CHER trial enrolment at <12 weeks of age, could be predictive of clinical, virological or immunological failure has been explored using a case-control analysis approach. Children from CHER who reached the trial-defined endpoint were defined as “cases” and included if stored specimens were available at both enrolment and up to a month prior to reaching the endpoint. “Controls” were children who did not met an endpoint at any stage throughout the trial, adhered to their randomization arm and had stored specimens available at enrolment and age-matched to a “case” at the time of the “case’s” endpoint. The CHER trial endpoint was death or failure of first line ART, defined as CD4 <20%, HIV disease progression to CDC stage C or severe B, CD4% <20%, or regimen-limiting toxicity [68]. Cases and controls were matched by trial arm, gender, ethnicity and study site (PHRU or KIDCRU).

The immune parameters available from enrolment were examined using only the early ART arms (ART-40W and ART-96W combined). The analysis revealed a large number of immunophenotypes that were significantly different between “cases” and “controls” at enrolment. In “cases” there were significantly lower CD4% (paired T-test, p=0.009), lower CD4 count (p=0.03), higher CD8 count (p=0.007), lower CD4:CD8 ratio (p=0.001), lower CD56 count (p=0.004), higher memory CD8 count (p=0.02), higher activated CD8 count (p=0.0004), higher naïve CD8 proliferation count (p=0.02) and higher memory CD8 proliferation count (p=0.04; Figure 4.1).

Figure 4.1: Immunophenotypes that significantly differ in CHER case-control analysis.



"Cases" are children who reached a trial-defined primary endpoint: death, failure of first line ART, progression to CDC stage C or severe B, CD4% <20%, or regimen-limiting toxicity. "Cases" (red) and "controls" (gray) are compared at CHER trial enrolment and time of trial-defined endpoint in the early ART groups combined (ART-40W and ART-96W). Pairs of cases and controls at enrolment: ART-40W n=20, ART-96W n=17; and at trial-defined endpoint: ART-40W n=18, ART-96W n=12. Key: aCD8 = activated CD8 T-cells, mCD8 = memory CD8 T-cells, mCD8 Ki67 = proliferating memory CD8 T-cells, nCD8 Ki67 = proliferating naïve CD8 T-cells. All comparisons are by Welch's t-test and p-values are all <0.05 in this figure.

Univariate logistic regression was used to model the relationship between each immunological parameter and the probability of reaching a trial-defined endpoint (clinical, virological or immunological failure, or death). Table 4.4 presents these data with "cut-off values" which suggest the level of an immune parameter to give 80% or 95% probability of reaching a trial-defined endpoint in children who have commenced ART at <12 weeks.

Immunological markers from around the time of reaching trial endpoints were also examined between "cases" (children reaching endpoints) and "controls" in the early ART arms and the following significant differences were identified in cases: lower CD4% (t-test, p=0.002), higher CD8% (p=0.003), higher CD8 count (p=0.03), higher

activated CD8 count (p=0.03) and lower CD4:CD8 ratio (0.006) (Figure 4.1).

Table 4.4: Immunophenotypes at enrolment that might be predictive of a trial-defined endpoint. Levels of lymphocyte subsets measured from ART-40W and ART-96W combined at trial enrolment (n=74) that have 80% or 95% probability of reaching a trial-defined endpoint (clinical, virological or immunological failure, or death). Counts are in cells/μl blood.

	Endpoint probability	
Immune parameter	**>80%**	**>95%**
CD4% of all lymphocytes	<28.5%	<23.5%
CD4 count	<570	<495
CD8 count	>2800	>3300
CD56 count	<135	<110
Activated CD8 count	>1750	>2200
Memory CD8 count	>690	>860
Memory CD8 proliferation count	>335	>430
CD4/CD8 ratio	<0.72	<0.61

Immunological markers before and after an episode of pneumonia in CHER participants were examined ("pneumonia-cases") and compared with age- and arm-matched children who did not have pneumonia ("pneumonia-controls"). "Pneumonia-cases" had lower CD4% and total count, and CD4/CD8 ratios before and after pneumonia (paired T-test p=0.0004, 0.0009 and 0.02 respectively combining pre- and post- measures) compared to "pneumonia-controls" despite small numbers (n=14 pairs of "pneumonia-cases" and "pneumonia-controls"). Before "pneumonia-cases" had lower naïve CD4, higher memory CD4 T-cells and higher activated CD4 T-cells (non-significant trend T-test p=0.06, 0.06 and 0.07); and after "pneumonia-cases" exhibited higher naïve CD4 proliferation, higher memory CD8 and lower naïve CD8 T-cells, and less CD3 T-cells overall (T-test p=0.003, 0.02, 0.02 and 0.09) than their respective controls.

4.3 Discussion

Immunological responses to HIV and ART are described in detail in this chapter to further understand the immune dynamics of HIV infection. During the first 4 months of life HIV-infected children starting ART, compared to HIV-uninfected children, had a host of immune disturbances: lower CD4/CD8 ratio, higher CD8 subsets including activated CD8 T-cells, NK-cells, naïve B-cells and increased proliferation of all of the above mentioned immunophenotypes. Thereafter, rates of proliferation decline with time on ART and most lymphocyte subsets return to what is considered normal in healthy South African children, however on interrupting ART many of the immunological responses seen prior to ART-initiation reoccur. However, when ART is reinitiated the changes do not appear to normalise entirely and further analysis revealed numerous immunological disturbances for ART-96W on ART up to 8 years of age, despite prolonged duration of ART. On-going activation and cytotoxic activity despite clinical stability on ART may be caused by immune-activation, low-level viral replication and/or co-infection that is more common in an immunocompromised child.

These findings are supported by the analysis comparing ART-Def versus ART-96W, and ART-40W versus ART-96W demonstrating that ART-Def off ART especially in the first 8 months of life, and ART-40W while interrupting ART had greater immunological disturbances (increased cytotoxic T-cells, proliferation, activation and decreased naïve T-cells, natural killer cells and CD4/CD8 ratio) than their counterparts on ART (ART-96W). Although these immunological disturbances exist while off ART, the differences tend to resolve when ART resumes, although as shown previously not to equal those of HIV-uninfected children. Due to the ART-strategies of the CHER trial, it is not known whether early and continuous uninterrupted ART might preserve and maintain an immunophenotypic profile that may not differ from that of an HIV-uninfected child.

While the effect of ART-interruption was captured in ART-40W demonstrating disturbed immune dynamics, the immediate effect of interrupting ART after 96 weeks on the immunophenotypic profile has not been captured by the sampling strategy used here. Therefore it is not possible to say whether the same immunological disturbance would occur after interrupting ART following 96 weeks of ART rather than just 40 weeks. Never the less, by 3 and 5 years of age no differences appear to exist, inferring any differences might only be short-lived and that interrupting ART

after 40 weeks is probably not inferior to 96 weeks in terms of long-term immunological dynamics in the immunophenotypic profile measured here.

The finding of highly significant and constant low memory CD4 T-cells in ART-96W regardless of ART-status has been recognized elsewhere [30] but is difficult to explain. It could plausibly be due to increased trafficking of memory CD4 T-cells to lymphoid organs and/or potential low-level HIV RNA replication, infection and destruction of CD4 memory T-cells. Alternatively, it may not be a true finding due to the immunophenotyping strategy used since some memory CD4 T-cells express CD45RA but are negative for CD27, thereby underestimating the proportion of memory CD4 T-cells.

While several differences have been identified, unless the differences follow a particular trend, caution should be applied when interpreting multiple statistical comparisons as 1:20 tests could result as being significant by chance. In addition, the statistical difference may not always make biological sense or have clinical relevance. Further limitations of these findings exist because the analyses were done on cryopreserved PBMCs and the cryopreservation-thawing processes might have influenced the differences detected, although the differences in immunophenotypes do appear to follow a plausible pattern of being deranged while off ART.

Interestingly, the “endpoint-cases” and “pneumonia-cases” did not demonstrate concurrent responses. The immune profiles from the “endpoint-cases” prior to their trial-defined endpoint demonstrated increased activated, memory and total CD8 T-cells alongside proliferation of both naïve and memory CD8 subsets, and decreased NK-cells and CD4/CD8 ratio. Decreased CD4/CD8 ratios and higher memory CD8 were also seen in the “pneumonia-cases”, however in contrast to the “endpoint-cases”, higher naïve CD4 proliferation and memory CD4 were shown. The responses are largely from CD8 T-cells in the “endpoint-cases” and likely reflect cytotoxic activity directed at virally infected cells, whereas the “pneumonia-cases” mostly reflect T-helper responses presumably from encountering new antigen from the intercurrent infection with increased proliferation of naïve CD4 to compensate for the switch from naïve to memory CD4, or perhaps clonal proliferation of a restricted T-cell receptor reservoir, which would increase susceptibility to intercurrent infection.

The endpoint case-control framework permitted the analysis of immune profiles at enrolment to determine which cell-surface markers measured at <12 weeks of age might be predictive of poor clinical outcome. This information could be valuable for clinical management by identifying children at risk and providing more individualised care for example frequent follow-up, prophylactic antibiotics or nutritional supplementation, however such a tool may not be feasible for children in Africa were access to diagnostics are limited.

The consequences of these immunological disturbances, particularly an on-going proliferative state are not fully known although it is plausible that there may be loss of functionality, immune exhaustion and increased apoptosis further driving proliferation. This raises the question of whether suppression of HIV RNA should not be the sole goal of therapeutic management in HIV-infected children and whether clinicians should also be aiming to normalise the immunophenotypic profile to optimise the immunological health of HIV-infected children; such approaches might include anti-inflammatories and broadly neutralising antibodies. Long-term studies of clinical and detailed immunological outcomes alongside molecular measurements of telomere length providing inferences on T-cell replicative histories may be of value here to further examine and understand the consequences of the immunological disturbances that have been seen here.

From a long-term perspective, despite the numerous potentially detrimental differences in immunophenotypes described in ART-96W compared to the CWC children, the findings of significantly higher naïve CD4 T-cells and total CD3 T-cells at 4-8 years in ART-96W is likely to reflect the long-term advantage of ART in augmenting thymic output beyond that of a HIV-uninfected child, as has been observed elsewhere [66]. While promising findings, these outcomes need to be consistent with improved clinical health for any real value to be placed upon them. This is generally true in CHER since ART-96W had less HIV-associated clinical events [68] however further longitudinal studies are required to establish this association in more detail.

Chapter 5

Thymic output in paediatric HIV

5.1 Background

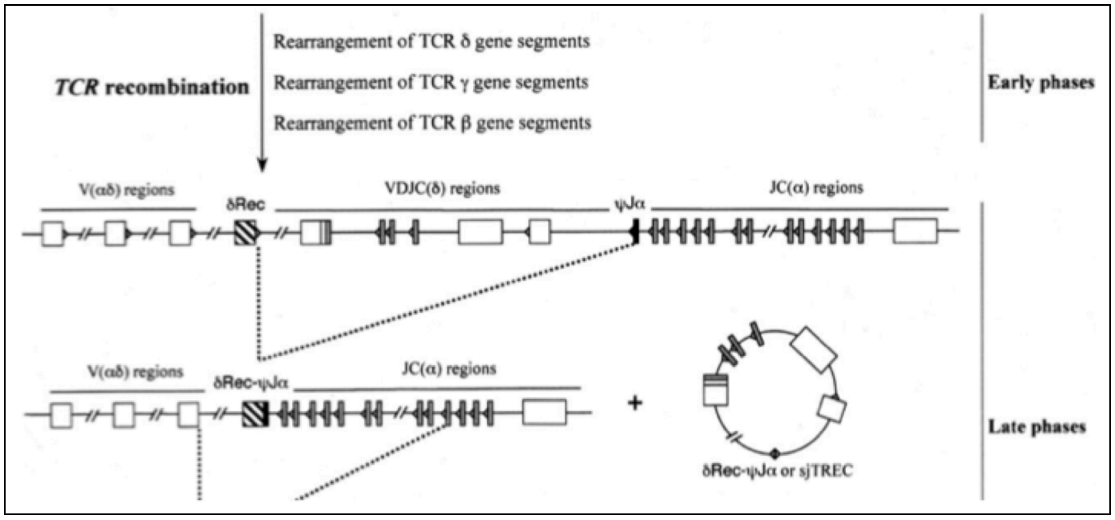
In this chapter the influence of HIV and antiretroviral therapy (ART) on thymic output in infants and children are examined. Thymic output is likely to have an important role in restoring the naïve CD4 T-cell pool following HIV-induced depletion, particularly so in early childhood years while a competent T-cell receptor repertoire is being established. However, there is currently limited understanding of the impact of HIV and effect of ART upon thymic output largely due to the difficulties involved in measuring thymic output.

There are several limitations in previous methods for reliably measuring thymic output. It is difficult to quantify thymic output due to it's inconvenient location in the anterior mediastinum, the variable rate of production of naïve T-cells [156], and lack of appropriate markers. In addition, numerous factors are presumed to influence thymic output and might affect long-term or day-to-day thymic activity. These include genetics, nutrition, hormones, vaccination, intercurrent infection and environmental exposure to microbes and allergens [157-160].

Attempts to quantify thymic output range from serial ultrasound scans, computed tomography (CT) and position emission tomography (PET) [161], to cell surface and intracellular markers detected by flowcytometry and molecular approaches; however all have their advantages and limitations [162]. Ultrasounds are cheaper and safer than CT and PET yet are dependent upon performance expertise and are no longer possible when the sternal bone has become calcified at approximately 2 years of age. Studies have used in vivo deuterium-labeled water (^2H_2O) to quantify the turnover rates of a range of immunophenotypic markers such as naive CD4 T-cells [163-165] since it is incorporated by new naïve T-cells that are produced in both the thymus and the periphery. Deuterium is safe at controlled doses but requires regular sampling so is impractical on a large scale and especially in paediatrics.

Immunophenotyping of circulating naïve T-cells is compromised by variable rates of peripheral expansion and/or lifespan, and previously used markers such as CD31 or PTK7 can also be markers of activation [166] or apoptosis [167] respectively. Molecular measurement of T-cell receptor excision circles (TRECs) by DNA PCR has been used marker of recent thymic emigrants [168]. TRECs are episomal DNA circles that are by-products of the rearrangement of the variable (V), diversity (D) and joiner (J) genes of the TCR α and β chains [63] (Figure 5.1).

Figure 5.1: Formation of T-cell receptor excision circles (TRECs), an episomal remnant of DNA that is spliced and excised during TCR gene rearrangement [63].



In healthy children and adults TREC levels decline with age [169] and thymic size; however, since TRECs are stable and do not duplicate during mitosis, they are diluted upon cell division making absolute measurements of TRECs potentially

misleading [84, 170, 171], especially in cases of increased peripheral proliferation as occurs in HIV-infection [81, 170, 172, 173]. These proliferative effects have been seen to directly correlate with total CD4 T-cell counts [174]. In one paediatric study, a decrease in TRECs has been observed over 96 weeks of ART in 33 children aged between 0.3 and 15.5 years [175], however this effect may be attributable to the reduction of peripheral T-cell death and cell-division secondary to ART-induced viral suppression.

The issue of TREC dilution upon cell-division may be avoided using a mathematical formula established by Bains et al [11, 82]. The formula combines TREC quantification with total body naïve CD4 T-cells and naïve CD4 T-cell proliferation using the marker Ki67 [174] (described in detail in Chapter 2, section 2.4.4) to create an explicit measure of thymic output expressed as the number of naïve CD4 T-cells exported from the thymus to the periphery per day.

This method was used in a recent study of 60 HIV-infected children and adolescents which suggested that age-adjusted thymic output is reduced in untreated HIV-infected children but significantly increases with proportion of life on ART [66]. “Proportion of life on ART” was used rather than “time on ART”, since children were of different ages at the time of measuring thymic output and ART was started at different ages too. Although limited by heterogeneity of age and ART history, this study importantly suggested that after starting ART, the thymus is able to at least partly compensate for the loss of naïve CD4 T-cells by increasing thymic output.

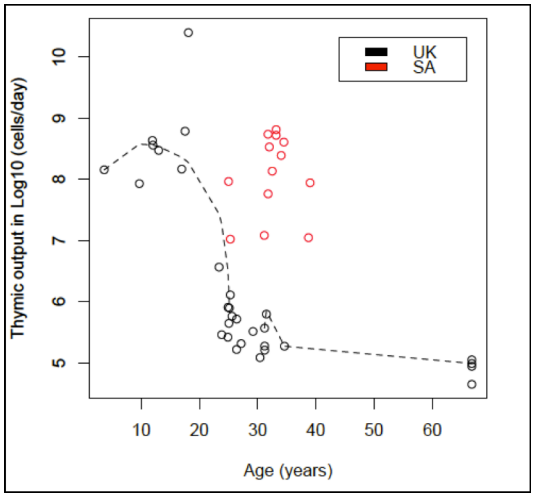
Therefore in this chapter, thymic output was estimated by combining the measurement of TRECs from purified naïve CD4 T-cells with quantities of naïve CD4 T-cells and rates of proliferation in the mathematical model established by Bains et al. Samples from healthy HIV-uninfected children at the Child Wellness Clinic (CWC) in South Africa were used to characterize and explore normal thymic output. Alongside CWC estimates of thymic output, a large number of stored PBMCs from HIV-infected children from the Children with HIV Early Antiretroviral Therapy (CHER) Trial were selected to measure thymic output enabling a comparison between children on ART with HIV-uninfected children. The estimates from the CHER trial also enable comparison of thymic output between the different CHER ART strategies, and the association between thymic output and clinical outcome.

5.2 Results

Thymic output preliminary work

Figure 5.2 illustrates declining thymic output across the age-range from 3 – 66 years for 43 individuals who participated in the preliminary work establishing this assay. These included 34 HIV-uninfected healthy adults (21 UK, 13 South African) and 9 HIV-infected children and adolescents from a London clinic. The South African measures of thymic output appear higher, however the PCR assay was performed on a different Taqman machine which uses an ultraviolet laser rather than an infrared laser therefore these results cannot reliably be compared.

Figure 5.2: Thymic output in Log10(cells/day) across age.



43 samples aged from 3-67 years (UK data: black circles, South African data: red circles). The regression curve is plotted through UK data only.

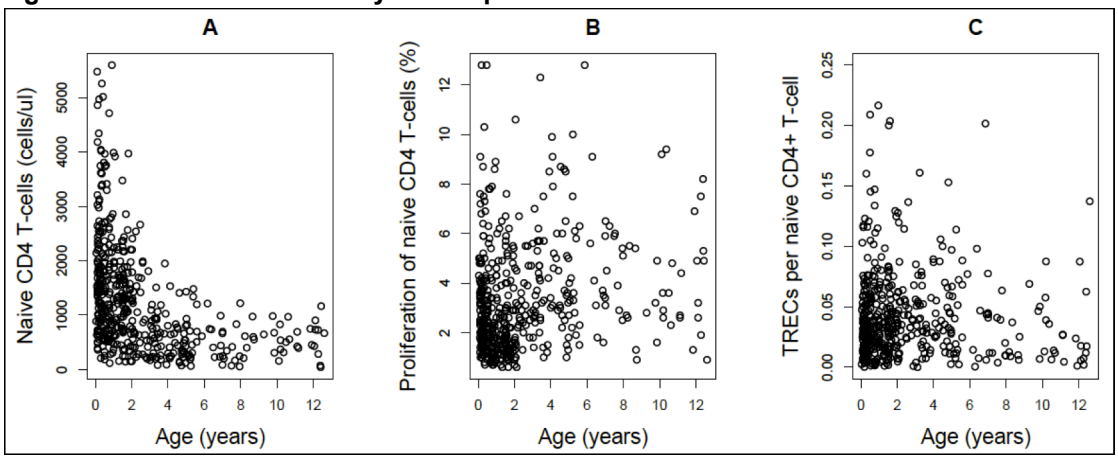
5.2.1 Thymic output in healthy HIV-uninfected children

454 samples were used to explore thymic output in healthy HIV-uninfected children from the Child Wellness Clinic (CWC), aged 2 weeks to 13 years. Each child contributed one measure of thymic output each to generate the normal range of thymic output in healthy South African children. The CWC structure and organization is described in detail in section 2.1 with the demographics and clinical characteristics of these children are described in section 3.2.1. All children with antenatal, neonatal and past medical histories (Table 3.1) were included in the "healthy" South African cohort with exception of maternal HIV-exposure (n=19). These subgroups have been

included since these are frequent conditions in this population and the children fulfilled the clinic recruitment criteria (currently well, no chronic medical condition, registered at the clinic, attended with biological mother and hand-held medical records). However each subgroup has been independently explored.

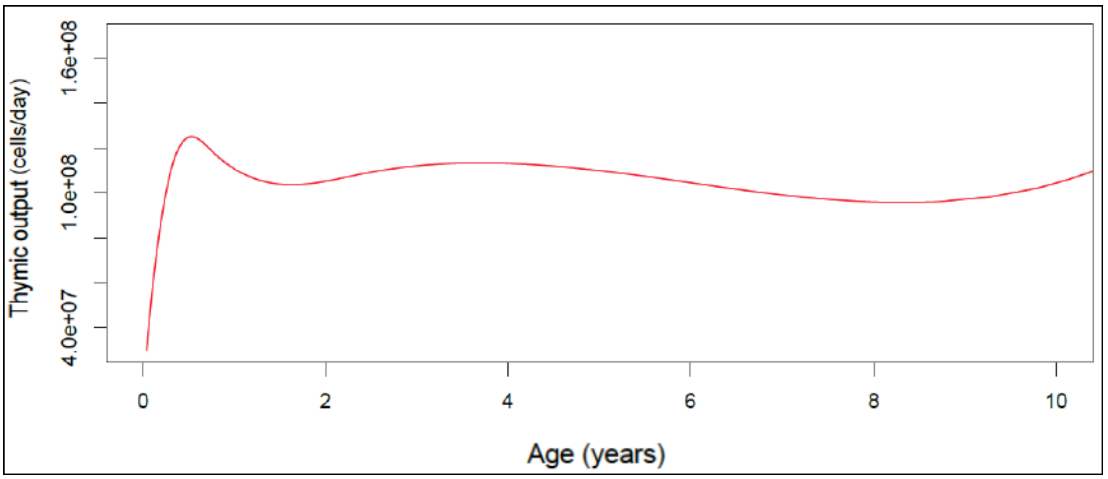
The elements of the thymic output model, described in section 2.4.4, are: total number of naïve CD4 T-cells per child, proliferation rates of naïve CD4 T-cells determined by Ki67 labeling and TRECs per naïve CD4 T-cell, and these are represented in Figure 5.3. These data combined in the mathematical model in Figure 5.4 demonstrate an increasing thymic output during the first 6 months of life with a characteristic peak between 6-12 months, followed by a decline until approximately 2 years and subsequent plateau throughout the remaining childhood years. However, in the first 2 years of life there is up to a 5-log variation in absolute thymic output measures (median $1x10^8$ [IQR $4.3x10^7$ to $2.7x10^8$] naïve CD4 T-cells per day. The distribution across age is illustrated in Figure 5.4, and Table 5.1 presents the data as reference ranges using the age-categories as used for the normal immunological parameters study and ranges derived from both the raw data and the exponential curve.

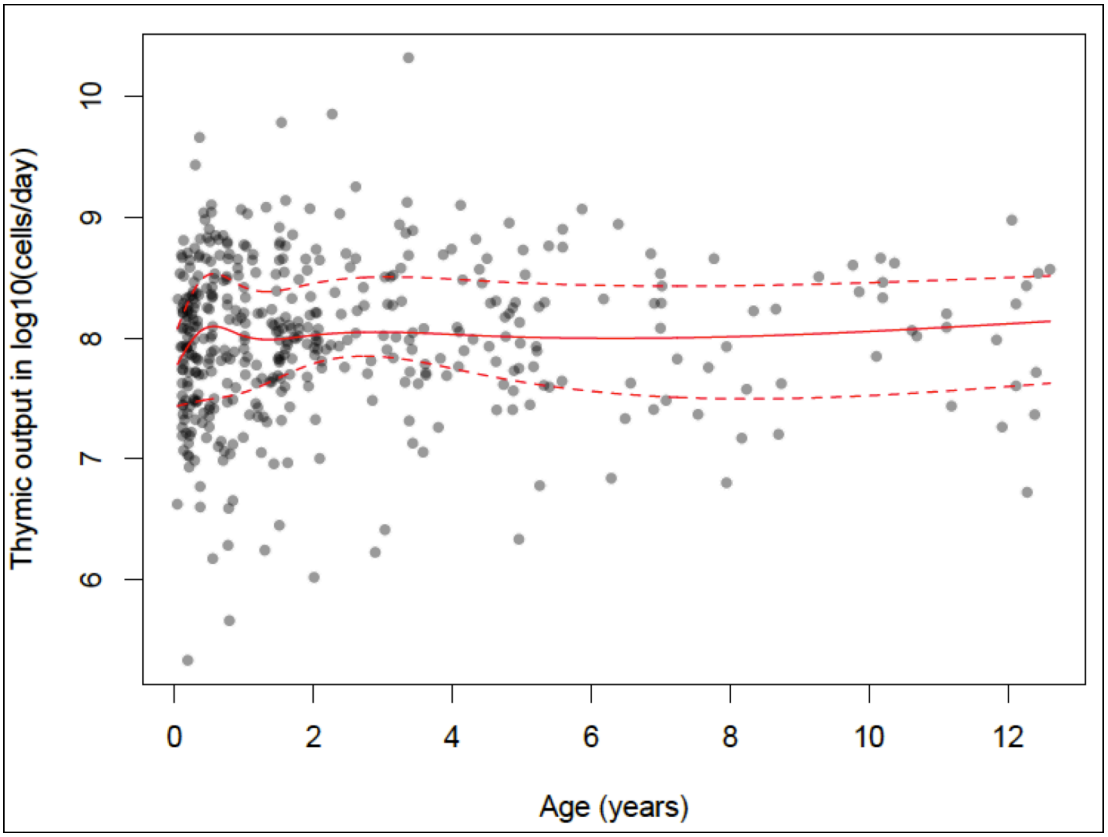
Figure 5.3: Elements of the thymic output model.



Plot A: Naïve CD4 T-cells, defined as CD3+CD4+CD45RA+ lymphocytes, in cells per microlitre of blood. Plot B: Proportion of Ki67+ naïve CD4 T-cells. Plot C: TRECs per naïve CD4 T-cell (maximum y-axis limited from 0.5 to 0.25 omitting 3 data-points to allow better visualization of the data).

Figure 5.4: Distribution of thymic output across age in healthy South African children from the CWC (Linear and Logarithmic scales).





The distribution is presented using non-linear regression to delineate the best fit of the data (n=435). Solid red line indicates median, surrounding dashed lines are 25th and 75th centiles. The non-linear regression curve was done with support from Dr Joanna Lewis (Methods section 2.5).

Table 5.1: Reference ranges for thymic output in healthy South African children from the CWC (n=435). The central value is derived from the non-linear regression curve, as illustrated in Figure 5.4.

Age-group	n	Log10 median [IQR]	Log10 mean (SD)	Log10 central value [95% CI]
0 – 3 months	63	7.9 [7.4 – 8.2]	7.8 (0.60)	7.9 [7.6 – 8.1]
3 – 6 months	52	8.1 [7.5 – 8.4]	8.0 (0.65)	8.0 [7.8 – 8.3]
6 -12 months	65	8.2 [7.5 – 8.5]	8.0 (0.75)	8.0 [7.8 – 8.3]
1 -2 years	87	8.0 [7.7 – 8.4]	8.0 (0.58)	8.0 [7.8 – 8.1]
2 – 6 years	119	8.0 [7.8 – 8.5]	8.1 (0.65)	8.1 [7.9 – 8.2]
6-13 years	49	8.1 [7.6 – 8.4]	8.0 (0.57)	8.1 [7.8 – 8.3]

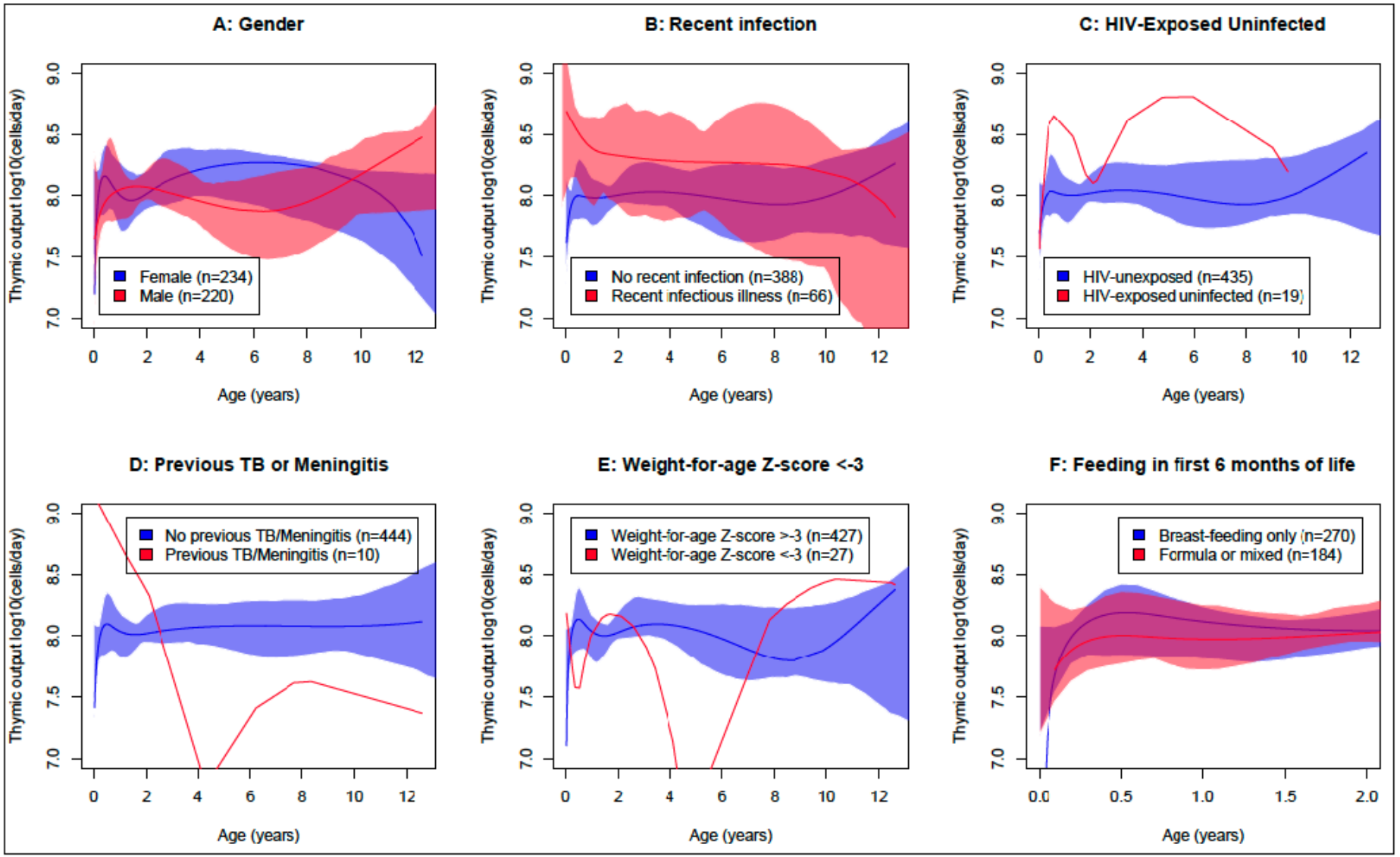
Demographic and clinical factors that might influence thymic output

The relationship between thymic output and numerous demographic and clinical characteristics recorded from the CWC were explored by comparing the regression curves from the subgroups of interest with superimposed regression curves of the children from the remaining population. A difference was determined by whether the best-fit curve of the subgroups of interest falls within the 95% confidence range of the remaining data. The characteristics and subgroups investigated were: gender (male 48%), ethnicity (black 22%), growth status (plateau growth n=23), extremes of weight-for-age Z-score (underweight z-score <-3 n=27, overweight z-score >3 n=6), immunisation status (not up-to-date n=72), feeding during first 6 months of life (breast, formula or mixed; n=270, 58 or 126), prematurity (<32 weeks, n=14), past medical history of meningitis or TB (n=10), maternal HIV-exposure but HIV-uninfected (n=19), and recent illness (1-4 weeks ago, n=66). The main variations found from the expected thymic output curve from all healthy children are presented in Figure 5.5. Otherwise if the sub-group best-fit median curves and 95% confidence intervals of the entire dataset largely overlap, it was presumed that no evidence of a significant difference was identified and the data are not represented.

Thymic output between male and female children does not appear to differ overall in the first 4 years of life. The male median curve does not feature the characteristic peak of thymic output in the first 6-12 months of life, however the 95% confidence interval for that line does capture the initial peak in thymic output. Between 4-10 years of age girls appear to have a higher thymic output than boys. Children with reported recent illnesses demonstrated higher thymic output than normal until 5

years of age, especially in the first year of life. The characteristic first peak of thymic output occurring at 6-12 months of age is much higher than the entire dataset in the small cohort of HIV-exposed uninfected children, and a second peak instead of a plateau follows the decline by 2 years. Similarly, the children who had a past medical history of either tuberculosis or meningitis had vastly different thymic output: far higher prior to 4 years and far lower thereafter. For children who had a less than or equal to -3 weight-for-age Z-score, the pattern of thymic output is erratic, fluctuating and difficult to interpret, but notable is the diminishing instead of increasing thymic output in the first 6 months of life. Although the confidence intervals of the two categories of feeding in the first 6 months of life largely overlap, breast-fed children had a higher peak of thymic output within the first year of life.

Figure 5.5: Subgroups of healthy children with different thymic output trajectories compared to the overall non-linear regression curve for the remaining healthy children. All x-axes represent age in years and all y-axes are measures of thymic output in Log10(cells/day). Plot A: Gender (male n=220 red; female n=234 blue); Plot B: Recent infection in past month (recent infection n=66 red; no recent infection n=388 blue); Plot C: Maternal HIV-exposure (HEU n=19 red, non-HEU n=435 blue); Plot D: Previous TB or meningitis (n=10 red; remaining children n=444 blue); Plot E: Weight-for-age Z-scores <-3 (n=27 red; remaining children n=427 blue); and Plot F: Feeding in first 6 months of life (breast n=270 blue; formula or mixed n=184 red). In all plots the central line is the median of each data set and the surrounding shaded area represents 95% CI for that line where sufficient data allowed. It was not possible to calculate 95% CI for Plots C-E since insufficient data were available within the subgroup.


A: Gender
Thymic output log10(cells/day)
Female (n=234)
Male (n=220)
Age (years)
B: Recent infection
No recent infection (n=388)
Recent infectious illness (n=66)
C: HIV-Exposed Uninfected
HIV-unexposed (n=435)
HIV-exposed uninfected (n=19)
D: Previous TB or Meningitis
No previous TB/Meningitis (n=444)
Previous TB/Meningitis (n=10)
E: Weight-for-age Z-score <-3
Weight-for-age Z-score >-3 (n=427)
Weight-for-age Z-score <-3 (n=27)
F: Feeding in first 6 months of life
Breast-feeding only (n=270)
Formula or mixed (n=184)

5.2.2 Thymic output in HIV-infected children on ART

HIV-infected children on ART versus to HIV-uninfected healthy children

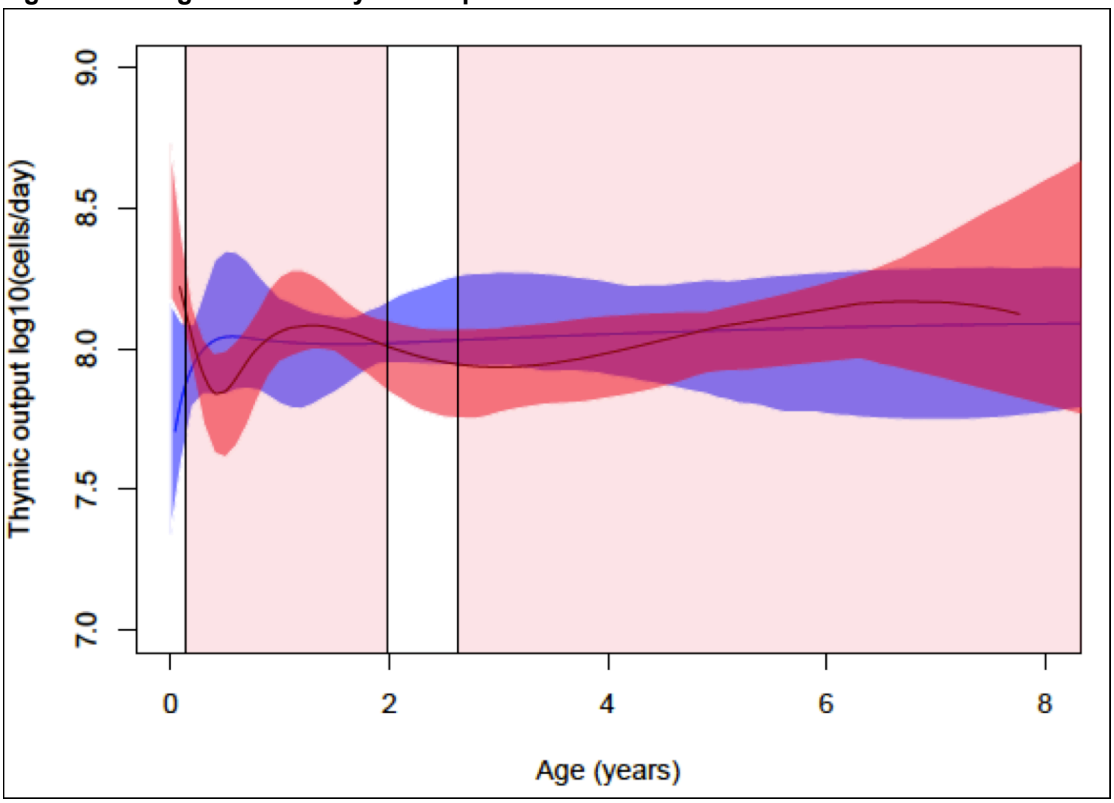
Measures of thymic output at single time-points in HIV-infected infants on early ART (CHER trial arm: ART-96W, n=321 from 4.3 weeks to 7.8 years of age) have been compared to measures of thymic output from CWC HIV-uninfected infants and children (n=435 from 2 weeks to 8 years). ART-96W infants started ART at CHER trial enrolment at 7.4 weeks (IQR 6.4 - 9) and continued ART for 96 weeks; then as per trial ART-strategy these children underwent ART-interruption for a median for 70 weeks (IQR 35-109) resuming ART if clinical/immunological progression occurred. Regression curves of all available data from both cohorts were superimposed (Figure 5.6). Although there's no overall difference and substantial overlap with the confidence intervals, it does seem that a different pattern of thymic output is occurring. Dips in thymic output may correspond with the effect of not being on ART until 7 weeks of age, and ART-interruption that occurred at approximately 2 years of age. Since average duration of interruption for the children examined was 34 weeks, this might roughly correspond with rising thymic output after recommencing ART. The confidence interval for data between 7-8 years of age is wider since less samples were available at this age.

In the thymic output curve for healthy children every sample used from the CWC was from a different child, however in ART-96W 321 samples were used from 107 children. To assess whether the presence of duplicates affected the shape and magnitude of the thymic output curve duplicates were removed at random and the curve was re-plotted, demonstrating minimal change in the shape and quantity of thymic output overall for the line of best-fit and surrounding 95% confidence intervals (Figure 5.7).

To examine the differences in thymic output as illustrated by these regression lines, the “difference-from-expected thymic output” of each ART-96W child was calculated by comparing individual values of ART-96W thymic output with the regression curve made from all CWC children at the precise age of interest. Since CHER measurements of thymic output have been made at 4, 8 or 12 weekly intervals corresponding to their clinical visits during the trial, the “difference-from-expected thymic output” at these time-points have been examined in Table 5.2. Therefore, the significant differences from expected are demonstrated at 7 (p=0.003), 19 (p=0.05), 67 (p=0.02) and 140 (p=0.04) weeks of age. For the children from ART-96W, this corresponds with initiation of ART (at 7 weeks), probable viral load suppression (19

weeks), after approximately one year of viral suppression on ART (67 weeks), and approximately a year after ART-interruption (140 weeks).

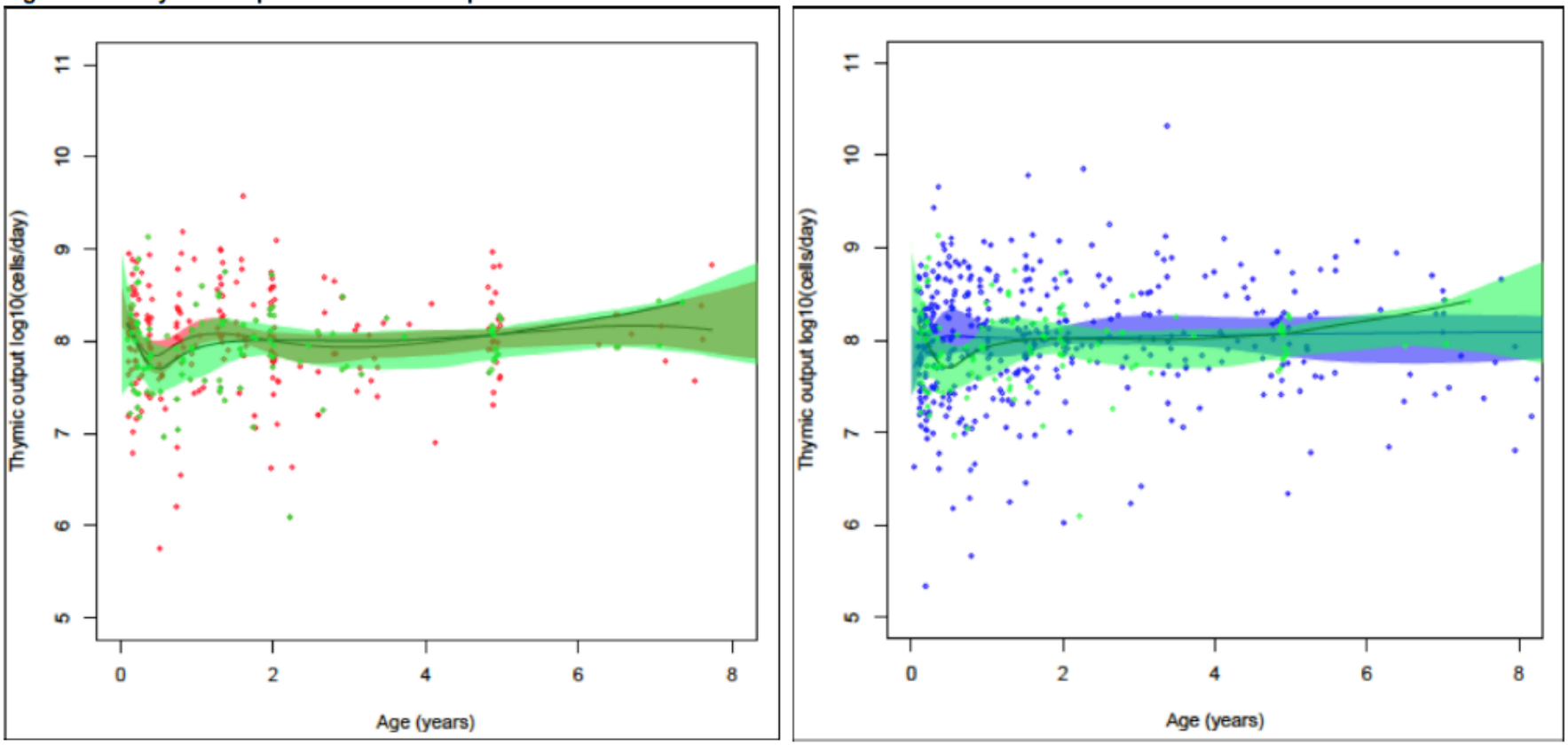
Figure 5.6: Regression of thymic output of CWC versus ART-96W.



Healthy children from the CWC with 95% CIs of the central value (blue line and surrounding blue borders, n=435) with best-fit regression are compared to 95% CIs of ART-96W (pink line with surrounding pink borders, n=321 samples from 107/144 (74%) of children from ART-96W). ART-96W commenced ART at median 7 weeks of age, continued for 96 weeks, followed by ART-interruption for median 34 weeks (IQR 0-152) for these 107 individual children. Pink shaded areas indicate time on ART for ART-96W.

Where longitudinal data were available, the pattern of thymic output illustrated by the ART-96W curve in Figure 5.6 was reflected by the profiles of individual children (Figure 5.8). Although there is a great deal of variability, thymic output tends to fall in the first 24 weeks then resurge or normalise thereafter.

Figure 5.7: Thymic output curves with duplicates in ART-96W removed at random.

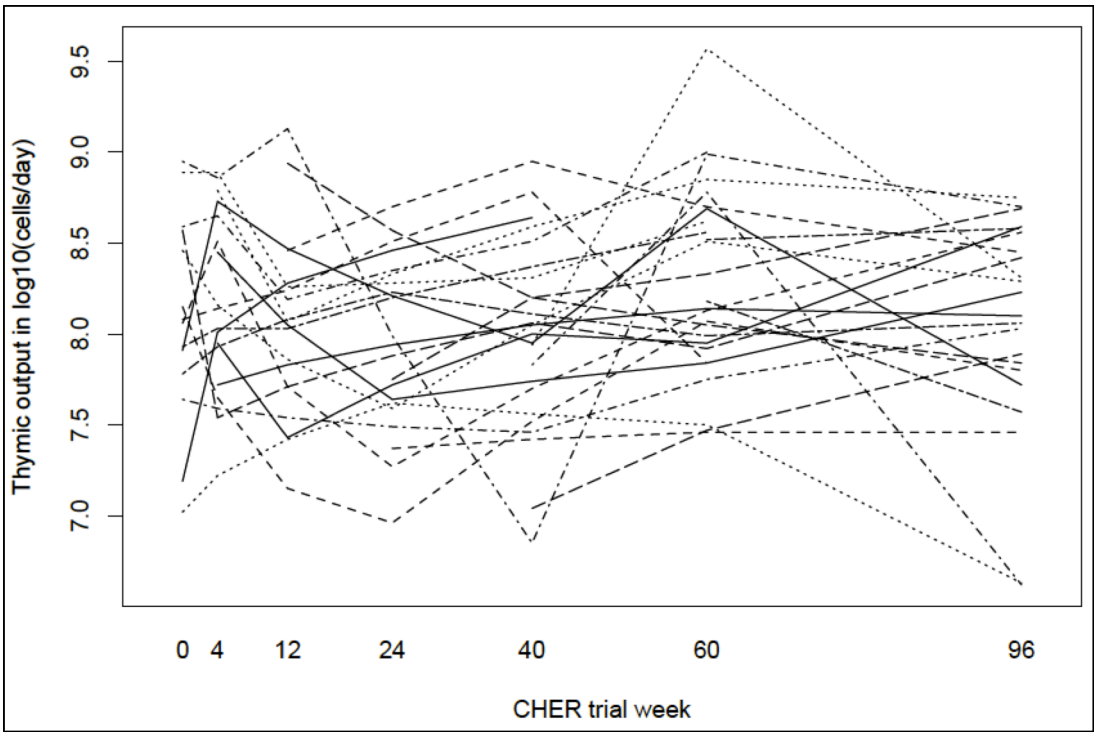


X-axis is age in years and y-axis is thymic output in Log10(cells/day). In red is the thymic output curve for all 321 samples from 107 individuals in ART-96W, in green is the same population of 107 children with repeats removed at random, and in blue is the thymic output data from the healthy HIV-uninfected population from the CWC (n=435). Each dot represents one individual data point. The first figure illustrates minimal change in the shape and magnitude of the thymic output curve in the population with duplicates removed compared to the entire set of data; and the second plot demonstrates that the de-duplicated curve (green) differs from the healthy control population (blue) at approximately the same time-points seen with the whole data set (red), as seen in Figure 5.6.

Table 5.2: Difference-from-expected measures of thymic output from ART-96W using the CWC regression curve. ART-96W divided into age groups and compared with expected value (i.e. zero). *Indicates significance at ≤0.05.

Approximate age (age range in weeks)	Number per group	Median log difference from expected, [IQR]	T-test p-value
7 (4 – 8)	32	0.3 [0.19 – 0.67]	0.003*
11 (9 – 14)	39	0.07 [-0.4 – 0.46]	0.60
19 (15 – 30)	52	-0.25 [-0.5 – 0.14]	0.05*
47 (38 – 57)	49	-0.08 [-0.44 – 0.24]	0.22
67 (66 – 74)	26	0.2 [-0.002 – 0.52]	0.02*
103 (102 – 108)	29	0.04 [-0.18 – 0.36]	0.73
140 (115 – 165)	25	-0.22 [-0.4 – 0.07]	0.04*
192 (170 – 215)	10	-0.004 [-0.41 – 0.14]	0.20
256 (252 – 261)	35	0.04 [-0.19 – 0.19]	0.68
366 (327 – 404)	13	0.08 [-0.05 - 0.4]	0.14

Figure 5.8: Longitudinal profile of 27 individual children from ART-96W with 4-8 separate measures of thymic output. These children commenced ART at week 0 (median 7 weeks of age) and remained on ART until 96 weeks.



Impact of CHER ART-strategies: Deferred ART versus early ART

The effect of deferring ART (ART-Def) until clinical or immunological progression was examined in comparison to ART started within 12 weeks of birth (ART-96W) in age-matched groups at enrolment, 40 and 96 weeks of the trial. The difference between observed and expected thymic output was compared ("difference-from-expected" thymic output) i.e. the difference between each individual CHER participant's thymic output and what would be expected for a normal South African child at that exact age as derived from the CWC regression curve.

No significant difference in "difference-from-expected" thymic output between ART-Def and ART-96W were identified at enrolment (n=35 vs. 40, Welch's t-test difference-from-expected Log10(thymic output in cells/day) p=0.51), 40 CHER trial weeks (n=39 or 31, p=0.41) or 96 weeks (n=38 vs. 44, p=0.09; Figure 5.9A). Of the 39 children from ART-Def at 40 weeks, only 9 (23%) remained off ART and 30 had commenced ART albeit at different trial weeks as per initiation criteria or DSMB recommendation. The difference-from-expected thymic output at 40 weeks was not significantly different between ART-96W on ART (n=31), ART-Def on ART (n=30), and ART-Def off ART (n=9; ANOVA p=0.61), although ART-Def off ART demonstrated the lowest median estimate of thymic output (7.98 Log10(cells/day)). Similarly at 96 weeks of the trial, only 3 (7.9%) of the 38 ART-Def available at this time-point remained off ART and 35 had commenced ART albeit again at different trial weeks. Once more, no significant difference in difference-from-expected thymic output were detected between ART-96W on ART (n=44), ART-Def on ART (n=35), and ART-Def off ART (n=3; ANOVA p=0.16) although ART-Def off ART continued to demonstrate the lowest median estimate of thymic output (7.8 Log10(cells/day)).

Impact of CHER ART-strategies: ART-interruption after 40 or 96 weeks ART

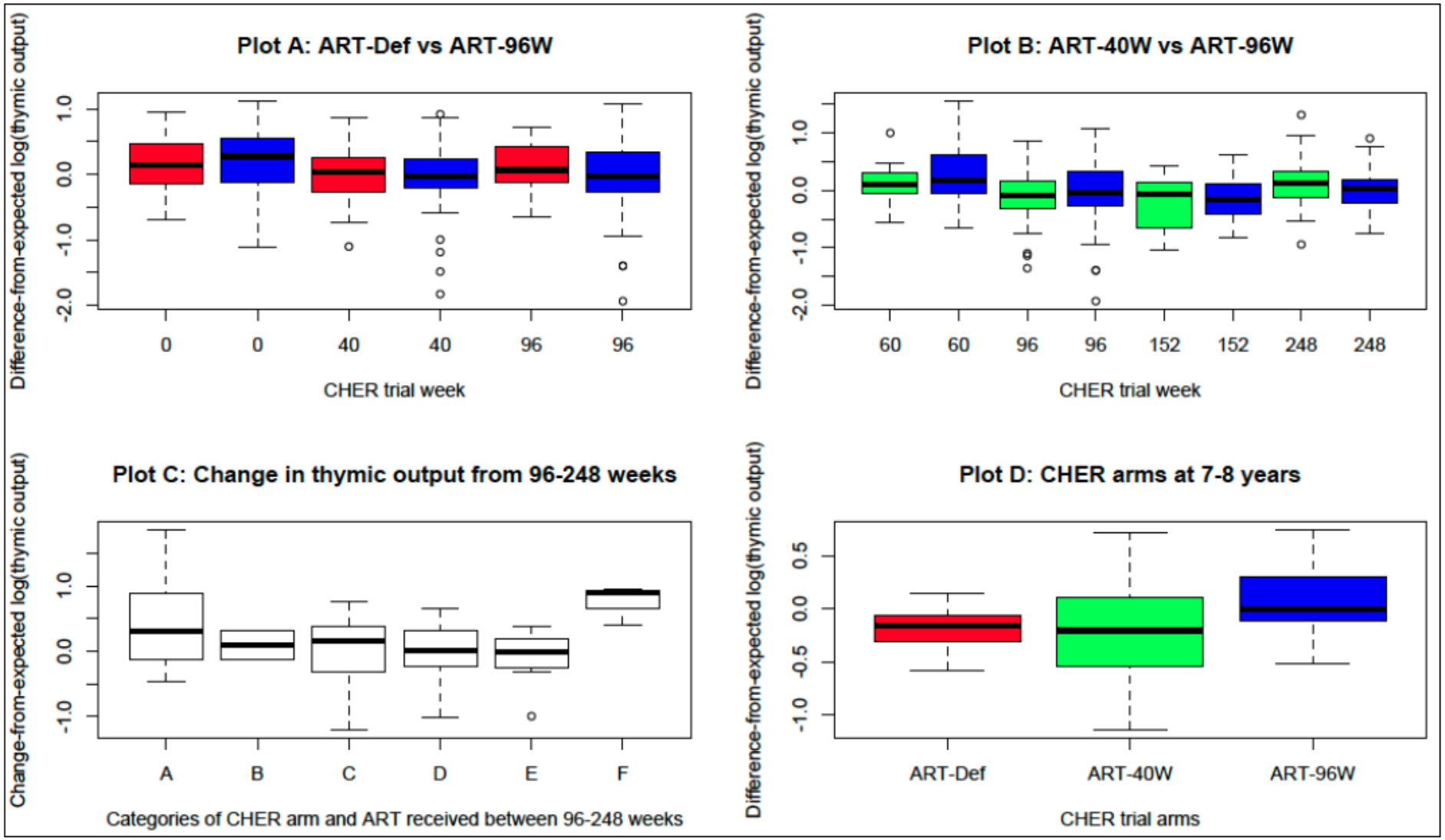
The effects of having a planned ART-interruption during the second (ART-40W) or third (ART-96W) year of life on thymic output were examined at 60, 96, 152 and 248 weeks of the CHER trial. No differences were found between the arms at each of the time-points examined (Welch's T-test difference-from-expected log(thymic output) n=32 vs. 35 p=0.11 at 60 weeks for ART-40W and ART-96W respectively; n=47 vs. 44 p=0.86 at 96 weeks; n=9 vs. 19 p=0.84 at 152 weeks; n=41 vs. 36 p=0.15 at 248 weeks; Figure 5.9B). Stratifying both arms by ART-status at each time-point (e.g. at 152 weeks four groups can be compared: ART-40W on or off ART and ART-96W on or off ART) still did not reveal any significant differences in thymic output. There was one exception: ART-40W off ART had significantly higher thymic output than ART-96W off ART at 248 weeks (n= 14 vs. 18, median 8.3 [IQR: 8.2–8.7] vs. 8.1 [7.8–8.2] Log10(cells/day), p=0.03) and higher than both ART-40W and ART-96W on ART by Welch's t-test comparing difference-from-expected log(thymic output) (p=0.04). Examining total duration of ART by trial week 248 in the early-ART arms (ART-40W and ART-96W combined) in children who recommenced ART did not demonstrate any correlation between cumulative duration of time on ART and thymic output.

Sixty-seven paired samples were available at both 96 and 248 weeks, therefore the size and direction of the change in thymic output, adjusted by what is expected for age, was examined in the early-ART arms, stratified by ART-status i.e. continuous ART, interrupted-and-remained-off ART or interrupted-and-recommenced ART between 96 and 248 weeks in ART-40W or ART-96W. In each of these subgroups there was no significant difference in thymic output identified with one exception: there were 3 pairs of samples from children who reached a trial-defined endpoint (clinical, immunological or virological), and these children did demonstrate a significant median increase of 1 Log10(cells/day) thymic output between 96 and 248 weeks (ANOVA p=0.02, Figure 5.9C).

Impact of CHER ART-strategies: Long-term

The long-term impact of the CHER ART-strategies on thymic output was compared difference-from-expected log(thymic output) at 7-8 years of age in all 3 arms: ART-Def, ART-40W and ART-96W (n=12, 21 and 11). ART-96W had higher than expected thymic output (mean 0.06 Log10(thymic output) higher than expected for age), SD 0.32) than ART-40W and ART-Def (p=0.08 and 0.04 respectively; Figure 5.9D). In absolute numbers this is 6.24 x 10^7 naïve CD4 T-cells/day more for ART-96W than healthy HIV-uninfected controls, 1.4 x10^7 more cells/day for ART-40W and 3.85 x 10^7 less for ART-Def. By 7-8 years, ART-96W had less time on ART compared to ART-Def and ART-40W (ANOVA 0.02), and 5 children from ART-96W remained off ART. These 5 children had a higher mean thymic output than expected for age (0.13 Log10(thymic output higher than expected for age (SD 0.36)) compared to children from ART-96W still on ART (n=8, mean 0.008 Log10(thymic output) higher than expected for age, SD 0.32) however this was not statistically significant (p=0.5). Examining the longitudinal perspective of thymic output from enrolment until 7-8 years of age in the combined early ART arms reveals sustained thymic output throughout at median 8 Log10(cells/day), (n=81 enrolment, 91 at week 96, 77 at week 248, 33 at week 360).

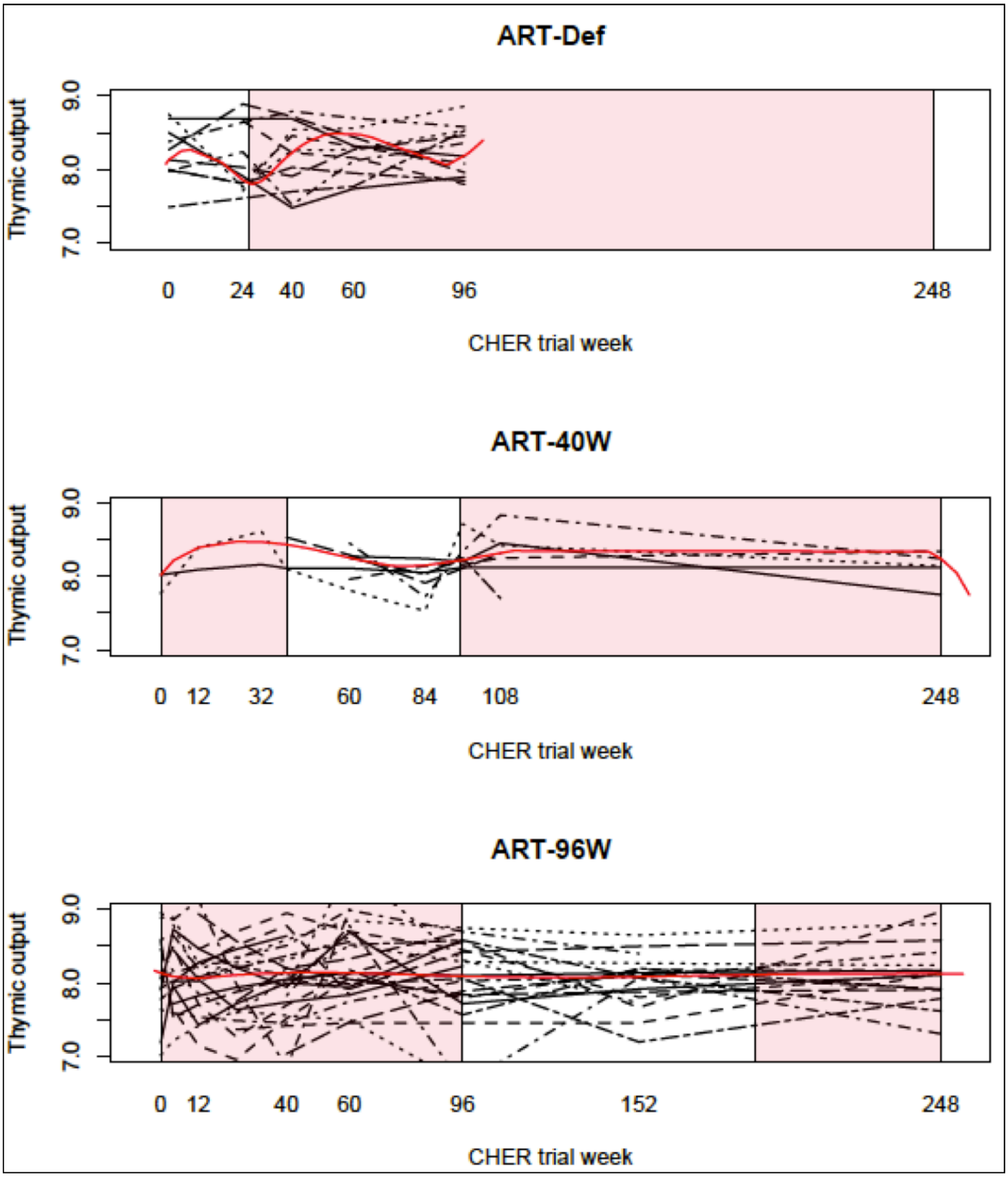
Figure 5.9: Effects of the CHER ART-strategies on thymic output. Plot A: Deferred versus early ART. No significant difference in difference-from-expected thymic output between ART-Def (red) vs. ART-96W (blue) at enrolment (n=35 vs. 40, Welch's T-test p=0.53), at 40 (n=39 or 31, p=0.41) or 96 weeks (n=38 vs. 44, p=0.09). Plot B: ART-interruption after 40 or 96 weeks of ART. No significant difference detected between ART-40W (green) vs. ART-96W (blue) at 60, 96, 152 and 248 weeks (n=32 vs. 35 p=0.17; n=47 vs. 44 p=0.86; n=9 vs. 19 p=0.82; n=41 vs. 36 p=0.15 respectively). Plot C: Change from 96 to 248 trial weeks between paired measures of thymic output in 6 scenarios of the early CHER arms stratified by ART-status (A=ART-40W off ART from 96-248 weeks, n=13; B=ART-40W off ART at 96 weeks but back on ART by 248 weeks, n=2; C=ART-40W on ART from 96-248 weeks, n=22; D=ART-96W off ART, n=17; E=ART-96W on ART, n=13; F=children who reached a primary endpoint from both ART-40W and ART-96W, n=3). F was the only group in which a difference was identified with a significant increase in thymic output from 96-248 weeks (ANOVA p=0.03). Plot D: Thymic output across all 3 arms of CHER at 7-8 years, ART-Def (red, n=12), ART-40W (green, n=21) and ART-96W (blue, n=11). ART-96W had a trend towards higher thymic output in direct comparisons with ART-Def and ART-40W (p=0.04 and 0.08).


Plot A: ART-Def vs ART-96W
Difference-from-expected log(thymic output)
-2.0
-1.0
0.0
1.0
0
0
40
40
96
96
CHER trial week
Plot B: ART-40W vs ART-96W
Difference-from-expected log(thymic output)
-2.0
-1.0
0.0
1.0
60
60
96
96
152
152
248
248
CHER trial week
Plot C: Change in thymic output from 96-248 weeks
Change-from-expected log(thymic output)
-1.0
0.0
1.0
A
B
C
D
E
F
Categories of CHER arm and ART received between 96-248 weeks
Plot D: CHER arms at 7-8 years
Difference-from-expected log(thymic output)
-1.0
-0.5
0.0
0.5
ART-Def
ART-40W
ART-96W
CHER trial arms

Trajectories of thymic output within individuals by CHER trial arm

Where available, multiple measures of thymic output within individuals allow the opportunity to examine the dynamics over time on and off ART (Figure 5.10). In the 12 children examined from ART-Def with multiple measures of thymic output, ART was commenced at mean of 26 weeks of the trial (range: 11.7-68.6 weeks). On examining the trajectory of thymic output 9/12 (75%, data not shown) of the children from ART-Def had low thymic output at the time of starting ART, and the regression curve of these results suggests thymic output then increases after initiation of ART. Although few data are available in ART-40W, thymic output appears to decline between 60-84 weeks after ART-interruption at 40 weeks, followed by an increase and plateau of thymic output corresponding to the mean ART-interruption of 55 weeks (range: 0-208 weeks). In ART-96W, within individuals there appears to be a decline in thymic output in the first 12 weeks, however this is not particularly evident from the central regression line which suggests a stable thymic output throughout at approximately 8 Log10(cells/day) despite ART-interruption after 96 weeks.

Figure 5.10: Profiles of thymic output from individual children within each CHER trial arm from enrolment to trial end (248 weeks).



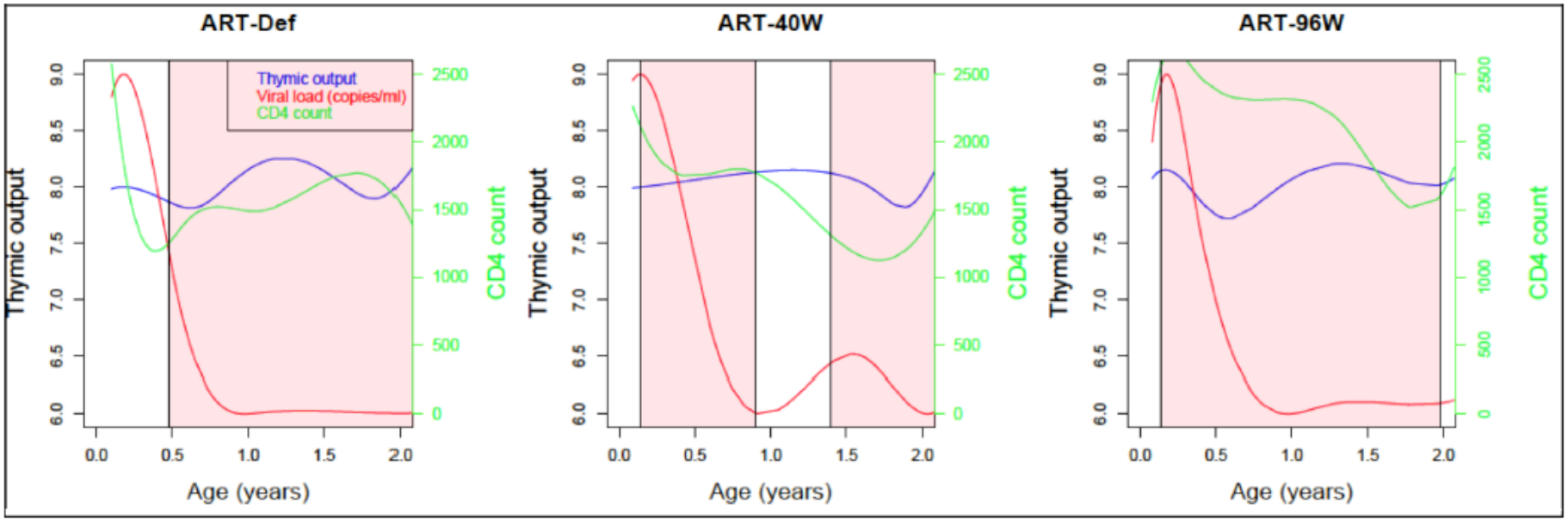
ART-Def (n=12), ART-40W (n=7), ART-96W (n=27); no children who reached the primary endpoints in the trial were included in these trajectories to illustrate the range and pattern of thymic output in clinically stable HIV-infected children and the impact of the CHER ART strategies used. The y-axes represent thymic output in Log10 naïve CD4 T-cells per day. The red line depicts median regression throughout data available. Shaded pink areas are time on ART: for ART-Def mean time to initiating ART for these 12 children was 26 weeks; for ART-40W mean ART-interruption after 40 weeks of ART was 55 weeks for these 7 children; and for ART-96W mean ART-interruption after 96 weeks of ART was 93 weeks for these 27 children (NB. 14 of these 27 children remained off ART until the trial end).

Trajectories of thymic output, viral load and CD4 count by CHER arm

The relationship between thymic output, CD4 and viral load has been explored within arms using regression curves (Figure 5.11). Available viral load and CD4 data were only included from children with corresponding measures of thymic output at that time, and therefore is not representative of the entire cohort. In all arms, viral load is suppressed within a year and remains suppressed in ART-Def and ART-96W until 2 years, however in ART-40W there is a viral resurgence coinciding with the period of time when ART was interrupted. CD4 count declines with age as expected, however the decline is more dramatic in ART-Def compared to ART-96W or ART-40W during the period where ART had not yet been initiated in ART-Def. ART-Def does appear to regain CD4 count to equal that of ART-96W by 2 years of age, whereas CD4 is lower in ART-40W, as expected as these children interrupted ART at week 40. Never the less, there were no significant differences in CD4 counts by 7-8 years between all arms (ANOVA, p=0.06).

In ART-Def and ART-96W there appears to be evidence of declining thymic output in the first months of life, followed by an increase to around 8 Log10(cells/day) for all arms at 2 years of age. In both ART-Def and ART-96W, increasing thymic output occurs following viral load suppression and is also associated with a plateau in the CD4 count. Very few data were available for ART-40W in the first few months (Chapter 2, Table 2.1: Specimen numbers), so the lack of decline in this arm is unlikely to be reliable, especially since the ART-strategy was as that of ART-96W in the first year of life. Correspondingly, in ART-40W at ~1.5 years of age following ART-interruption, there is a surge in viral load corresponding to a trough in thymic output and CD4 count.

Figure 5.11: Trajectories of thymic output with corresponding viral load and CD4 count from enrolment to 2 years of age.

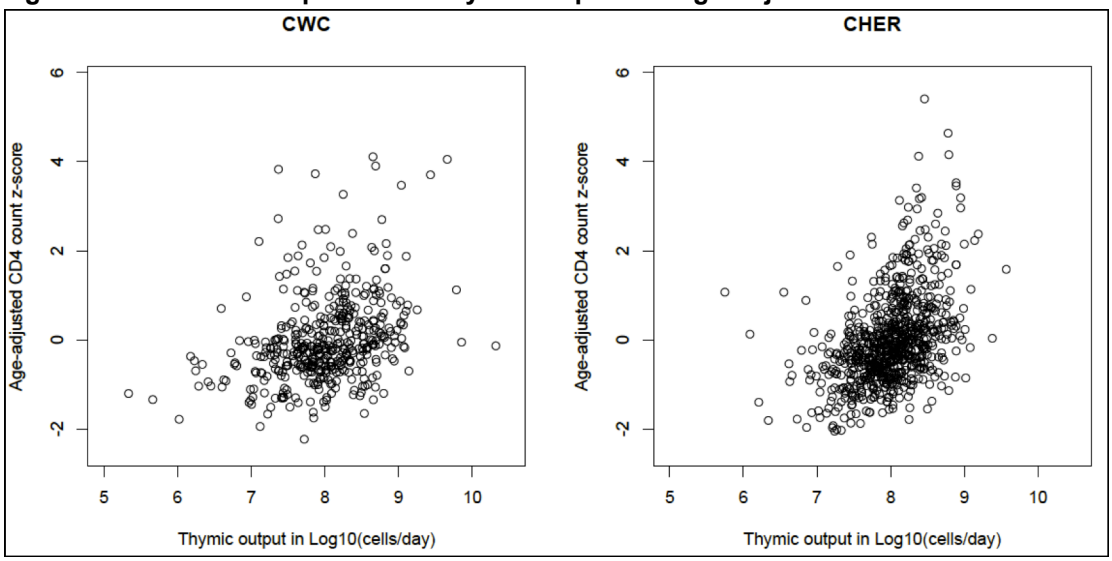


ART-Def (176 measures of thymic output in 80 individuals), ART-40W (190 measures of thymic output in 104 individuals) and ART-96W (250 measures of thymic output in 102 individuals). Thymic output in Log10 naïve CD4 T-cells/day (blue), CD4 count in cells/μl (green) and Log10 viral load in copies/ml (red, axes not shown, max 750,000 – min 399 copies/ml). Shaded pink area reflects time on ART: in ART-Def median time to starting ART in these 80 children was 24.9 weeks (IQR 20-39.3); in ART-40W median age of ART initiation was 7.5 weeks (IQR 7-9) in these 104 children with median 26 weeks ART interruption (IQR 9.4-100); and in ART-96W median age of ART initiation was 7.5 weeks (IQR 6.6-8.7) in these 102 children.

Thymic output and CD4 count

Simple linear regression was used to further explore the relationship between CD4 count and thymic output, and a significant regression equation was found for both CWC and CHER participants (all CHER participants were combined). Age-adjusted CD4 Z-score was calculated for each individual from either CWC or CHER against expected CD4 counts for healthy South African children using the CWC data-set (Statistical methods section 2.5: Thymic output). For CWC the parameters for linear regression are as follows: $F(1,450)=58.8$, $p<0.0001$ with $R^2=0.11$ and for CHER: $F(1,774)=188.7$, $p<0.0001$ with $R^2=0.2$. For CWC predicted CD4 Z-score is equal to 8+0.22(thymic output in Log10(cells/day)), or for CHER 8+0.21(thymic output). This means that CD4 count Z-score increases by 0.22 for CWC or 0.21 for CHER children, for each increase of Log10 cells/day of thymic output (Figure 5.12).

Figure 5.12: Relationship between thymic output and age-adjusted CD4 count Z-score.



CWC (n=452 children) and CHER (n=776 samples from 293 children). Age-adjusted CD4 Z-score was calculated for each individual from either CWC or CHER against expected CD4 counts for healthy South African children using the CWC data-set.

5.2.3 Unstable HIV-infection and thymic output

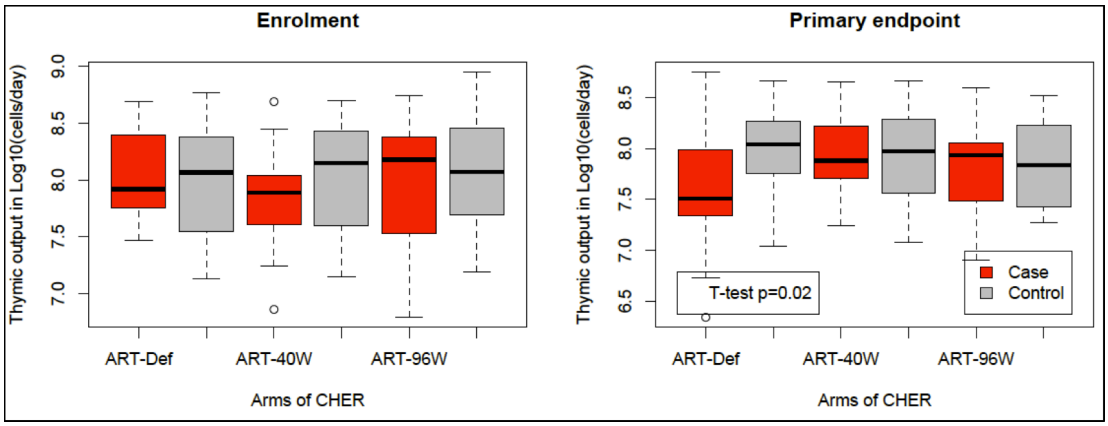
Thymic output in children who reached a CHER trial-defined endpoint

The CHER trial primary endpoint was death or failure of first line ART, defined as CD4 <20%, HIV disease progression to CDC stage C or severe B, regimen-limiting toxicity or CD4% <20% (increased to <25% in 2006 in line with international guidelines) [68]. The relationship between thymic output and trial endpoints has been explored in using a case-control analysis approach. Children who reached the trial-defined endpoint were defined as "cases" and included if stored specimens were available at both enrolment and up to a month prior to reaching the endpoint. "Controls" were children who did not meet an endpoint at any stage throughout the trial, adhered to their randomization arm and had stored specimens available at enrolment and age-matched to a "case" at the time of the "case's" endpoint. Cases and controls were also matched by trial arm, gender, ethnicity and study site (PHRU or KIDCRU). Controls were selected randomly within matched subgroups.

The case-control design at enrolment was used to assess whether measures of thymic output could be predictive of poor clinical outcome i.e. reaching one of the trial-defined endpoints. Although no significant difference was identified between each case-control pair, mean thymic output was lower in the cases of ART-Def and ART-40W at enrolment (Figure 5.13). Thymic output was next compared prior to or at the time of reaching the trial-defined endpoint between "cases" and "controls". Although no difference was seen in the early ART arms, ART-Def had significantly lower thymic output prior to reaching a trial-defined endpoint compared to the control group (n=20, t-test p=0.02; Figure 5.13).

Of the 52 children examined at enrolment who went on to reach trial-defined endpoints (i.e. the "cases"), the majority were due to death (n=26) or immunological failure defined as CD4 <20%/25% (n=17), however no significant relationship was demonstrated between thymic output at enrolment and going on to have immunological failure or die. Neither was a statistical relationship identified between thymic output at the time of reaching the trial-defined endpoint and type of endpoint in the 50 children examined.

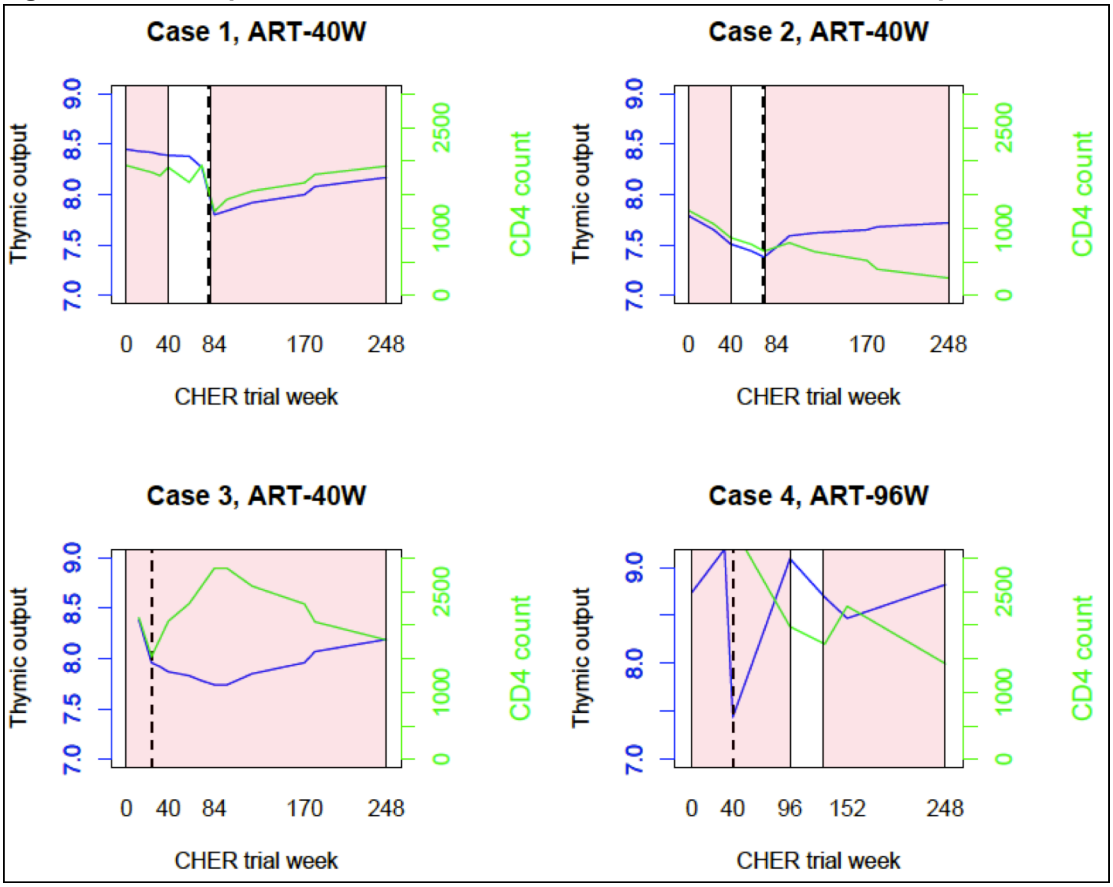
Figure 5.13: Case-control analysis of thymic output at enrolment and prior to reaching a trial-defined endpoint.



Each child reaching a trial-defined endpoint ("case", red) was age-matched to a "control" (gray) at the time of reaching the endpoint. Cases and controls were also matched by trial arm, gender, ethnicity and study site (PHRU or KIDCRU). Pairs of cases and controls at enrolment: ART-Def n=17, ART-40W n=20, ART-96W n=17. Pairs of cases and controls at the trial-defined endpoint: ART-Def n=20, ART-40W n=18, ART-96W n=12.

Nevertheless, examining the trajectories of thymic output in children with more than 4 individual measures of thymic output crudely revealed a pattern by which it appeared that thymic output was declining or at it's lowest for that individual at the time of reaching the primary endpoint. This was true in 8/12 (66%) of ART-Def, 4/5 (80%) of ART-40W and 2/2 (100%) of the "cases" examined in ART-96W. Figure 5.14 illustrates these findings in a selection of 4 individuals. Since "immunological failure" (CD4 <20%/25%) was one of the trial's endpoints, reaching a trial-defined endpoint corresponds with low CD4 count in 3 of the 4 cases. In case 1 and 2, the children underwent scheduled ART-interruption at 40 weeks, thereafter thymic output and CD4 count diminish and the trial-defined endpoint is reached around 80 weeks. In case 3, the primary endpoint was reached before ART-interruption occurred but coincides with low thymic output and CD4 count. As per trial protocol ART was subsequently not interrupted and thymic output eventually gradually increases on continuous ART. In case 4, the primary endpoint also occurred before ART-interruption with low thymic output although not low CD4 since this endpoint was reached by fulfilling clinical criteria (CDC severe B or C). However, ART was interrupted at 96 weeks and during ART interruption a fall in thymic output is evident.

Figure 5.14: Examples of 4 children who reached a CHER trial-defined endpoint.



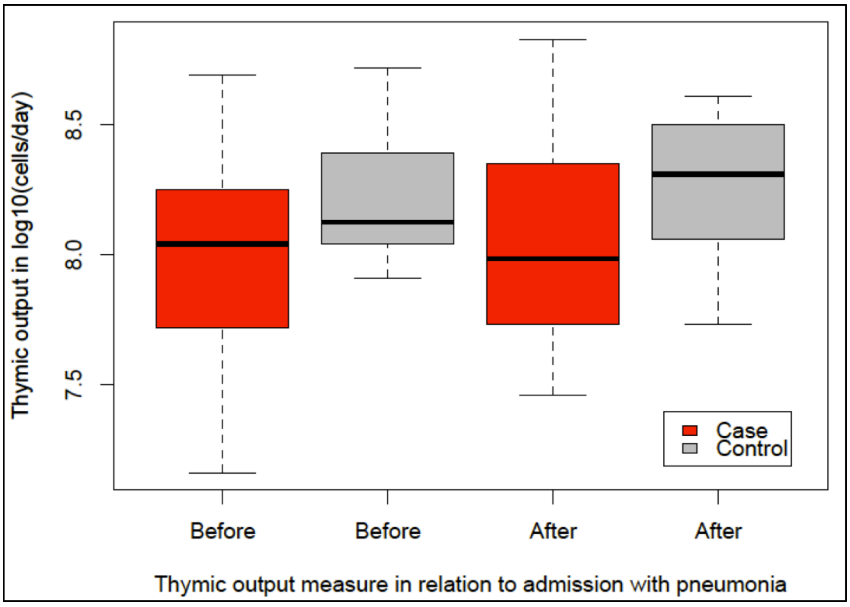
Individual trajectories of thymic output in Log10(cells/day) are marked in blue and CD4 count in cells/µl in green. The black dashed line indicates when the trial-defined endpoint was reached, and pink shaded area reflects time on ART.

The rate and direction of change in thymic output within individuals between pairs of thymic output measures at enrolment and endpoint were examined. The children who reached endpoints in ART-Def ("cases") demonstrated the greatest decline in thymic output that was significantly different compared to the age-matched controls from ART-Def (median decrease in "cases" -0.25 [IQR -0.41-0.03] Log10(cells/day/year) versus median increase in "controls" 0.13 [IQR -0.32-0.21], n=10 pairs of both cases and controls, t-test p=0.02). No significant difference was found in ART-40W (n=16 pairs), however in ART-96W a difference was seen in the change in thymic output over time between cases and controls, whereby the cases had more children who showed an increase in thymic output than in the controls, although median change was negligible in both groups (median change in "cases" -0.03 [IQR -0.38-0.4] Log10(cells/day/year) versus median change in "controls" -0.08 [IQR -0.67-0.02], n=12 pairs of both cases and controls, t-test p=0.04).

The effect of an intercurrent infection on thymic output

The effect of an intercurrent infection on thymic output was investigated by another case-control analysis examining the thymic output before and after an admission to hospital with pneumonia. The “pneumonia-cases” were defined by hospital admission for at least one night with an uncomplicated lower respiratory tract infection characterized by increased respiratory rate, chest recessions and oxygen requirement. Potential TB infections were excluded. “Pneumonia-cases” were matched to “controls” by gender, ethnicity, age, trial arm and trial site. “Pneumonia-controls” were stable HIV-infected CHER participants who had an uncomplicated medical history, were not admitted to hospital for pneumonia or any other issue, and did not reach a trial-defined endpoint at any stage throughout the trial. Measurements of thymic output were performed from cryopreserved PBMCs stored from clinic reviews before and after the admission. Fourteen pairs of pneumonia case-controls with pre- and post-admission measures of thymic output were available for analysis. Both measures of thymic output in “pneumonia-cases” were lower than controls although with a trend towards significance in pre-pneumonia measures (T-test p=0.09) and no difference in post-pneumonia (p=0.16, Figure 5.15).

Figure 5.15: Thymic output before and after admission with pneumonia.



Comparison of thymic output between “pneumonia-cases” (red) age-matched with well and stable CHER participants as “pneumonia-controls” (gray). Cases and controls were also matched by trial arm, gender, ethnicity and study site. Paired T-test pre-pneumonia p=0.09, post-pneumonia p=0.16, n=14 pairs of cases and controls.

5.3 Discussion

5.3.1 Thymic output in healthy HIV-uninfected children

The laboratory data from the CWC cohort were used to formulate a non-linear regression curve to delineate the change in thymic output during childhood. As was expected from the mathematical model, there was wide variability in thymic output values reinforcing the need to include large numbers to fully characterise the distribution. The laboratory data were similar to the model in demonstrating peak thymic output between 6-12 months of life followed by a decline until 2 years of age. However, thereafter our estimates appeared to deviate from the model's estimations by maintaining a constant thymic output into late childhood where the model demonstrated a gradual decline until teenage years and adulthood.

The reason for the discrepancies seen between the published model and our estimates of thymic output might be explained by a number of factors. The data used to form the model may have used different measurements for TRECs and Ki67 labeling, and were from a small number of children from a different study population in the US (n=12) and Europe (n=25). Therefore a number of variables may have been introduced such as genetics, nutrition and exposure to environmental pathogens and allergens; and since the variability is so extensive a few individuals with extreme measures might easily influence the original model. Although numbers from the CWC were large, they were from many different participants and longitudinal follow-up of individual children may have been preferable in delineating the thymic output curve.

A potential limitation of the thymic output model may result from the blood volume formula utilised to quantify the total number of naïve CD4 T-cells in the child's circulating blood. There are few formulas available to estimate blood volume in children; the most comprehensive study was performed by Linderkamp et al in 1997 [79] using a fixed ratio of plasma to red-cell volume (F-cell ratio) and weight to estimate blood volume and this approach has been used in estimating blood volume for thymic output in our study. Subsequent authors have suggested a fixed F-cell ratio is not plausible in children [176]; however better approaches to estimating blood volume are lacking. If available, a formula that reliably incorporated both weight and height may have been better since South African children could have stunted growth compared to their US or European counterparts used in the model.

Another limitation of the thymic output formula is that it is intrinsically constrained by an upper limit of 0.6 TRECs per naïve CD4 T-cell; however up to 2 TRECs per cell is biologically plausible if both alleles happen to have gene rearrangement [168]. The figure of "0.6" is derived from the average number of predicted cell divisions before a naïve CD4 T-cell leave the thymus. Although only 3 samples taken from the CWC had ≥0.6 TRECs per cell, these results had to be excluded since it would have produced an implausible value of negative thymic output. Thereby the robustness of the model is brought into question.

Finally, a further limitation of the model is the possibility that Ki67 may not only reflect increased levels of proliferation from a homeostatic response to low cell numbers but may be elevated by immune activation or intercurrent infection [163, 177], and may remain elevated for days after completion of cell-division [178], and therefore could give an inappropriately high estimate of thymic output.

A potential biological explanation exists for the lack of decline in thymic output after 2 years of age compared to what was predicted by the mathematical model. In Chapter 3, the CWC demonstrated a dramatic increase in the naïve/memory ratios of both CD4 and CD8 T-cells compared to industrialised countries, plausibly driven by environmental exposure to pathogens. Therefore the possibility of a feedback mechanism to the thymus is introduced, as increased antigen-stimulation converts naïve to memory CD4 and CD8 T-cells, the thymus may be mobilised to increase thymic export of naïve T-cells thereby maintaining a higher than expected thymic output, as seen by the CWC regression curve. In contrast, it is plausible that a more sterile environment lacking in antigen-stimulation, as occurs in the richer north, may play a role in reduced thymic output as seen in the published model.

This feedback mechanism to the thymus is supported by the exploratory analyses of the CWC subgroups, as children with common childhood illnesses within the past month manifested a higher thymic output than expected for age. This could be explained by the release of IL7 during infection, which is also recognized to promote maturation of thymocytes and is a known modulator of T-cell homeostasis [179]. Similarly, the HIV-exposed uninfected children, albeit a very small sample, had higher thymic output, potentially due to antigen-stimulation from circulating maternal antigens within breast milk, or increased environmental exposure since poorer socio-economic conditions have been associated with HIV-infected individuals [180].

Environmental pathogen exposure is unlikely to be the sole cause of the large variation in thymic output, and other factors have been implicated by the analysis of this study include gender differences, poor growth and method of feeding in the first 6 months of life. Gender differences might be due to differences in height not being accounted for using the blood volume formula, or pre-puberty hormonal changes. Likewise variable growth hormones and nutrition may explain erratic thymic output in children with poor growth; and finally breast-feeding might augment the peak thymic output in infancy which corresponds with reports of increased thymic size and improved immune responses in breast-fed babies [181]. However, these are all limited analyses and any potential associations require further investigation.

5.3.2 Thymic output in HIV-infected children on ART

Thymic output was compared between HIV-uninfected CWC children and age-matched HIV-infected children on ART from CHER (ART-96W). Although all measures of thymic output were derived from laboratory measures performed on cryopreserved PBMCs, CHER PBMCs had been stored in liquid nitrogen for up to 8 years compared to 6-12 months for CWC specimens, and some CHER specimens had low yield of live cells on thawing. In these specimens, it was the dead cells that appeared to have a higher proportion of activation and proliferation markers which might be due to activated cells being more prone to death during freeze-thaw processing, or the fact that dead cells can generate artifacts as a result of nonspecific antibody binding and unwanted uptake of fluorescent probes. Never-the-less, the fresh-frozen comparison described in Chapter 3 did not reveal a relationship between percentage of live cells and difference from the fresh measure of the immunophenotype of interest; therefore these samples were included in the analyses described in this chapter.

The confidence intervals of the CWC and ART-96W regression curves largely overlap, implying no significant difference overall between the two groups of children and that early ART might maintain thymic output to that of a healthy child. However, the trajectory of the median curve from ART-96W is quite different from the CWC and provides valuable information to help understand the initial response of thymic output to HIV, and then to treatment with ART. Adjusted analyses in Table 5.2 shows that the thymic output of children in ART-96W differs most from the CWC children at 7, 19, 67 and 140 weeks. At 7 weeks a higher thymic output in ART-96W might correspond with the thymus working hard to compensate for the loss of CD4 in the

early weeks of infection before ART had been initiated. There may be limited capacity for the thymus to maintain sufficient naïve CD4 T-cell output, since by 19 weeks lower than expected thymic output is seen despite these infants having received 3 months of ART. However, by 67 weeks, the viral load should have then been suppressed for more than a year and ART appears to have preserved thymic output or even increased it over that expected for a healthy HIV-uninfected child. In ART-96W, ART was continued for 96 weeks until ART-interruption for a median of 34 weeks in the children examined here, although it is a source of bias that there was a wide range ART-interruption duration with some children remaining off ART until the trial end. Nevertheless, thymic output falls during ART-interruption and is significantly different from expected for a healthy HIV-uninfected child at 140 weeks, subsequently rising gradually as children recommence ART.

As demonstrated by the ART-96W regression curve and the individual trajectories, falling thymic output in the first 3 months of life may reflect the detrimental effect of vertically-acquired HIV on the developing thymus both in-utero and perinatally before commencing ART. Infection of recent thymic emigrants with HIV has been demonstrated [182], however it is not known how this influences thymic output or affects the longevity and function of recent thymic emigrants. One limitation of comparing measures of thymic output between HIV-uninfected and HIV-infected children using the mathematical model is that the formula relies upon the assumption that 2% of lymphocytes reside in the blood. This may be true of HIV-uninfected children and for clinically stable HIV-infected children on ART [183], however it could be much lower in untreated HIV-infection [184]. In view of this, the formula would thereby underestimate thymic output in ART-96W at 7 weeks of age and the difference compared to CWC is likely to be greater and therefore more significant.

At 7-8 years of age ART-96W had better thymic output than ART-40W and ART-Def despite having received less cumulative ART overall. This is most likely to be due to the ART-strategy used; early-ART continued for 96 weeks rather than to only 40 weeks, and could suggest that early ART for at least 2 years may sustain a good level of thymic output for longer. This is reinforced by the CD4 and thymic output data from the CWC and combined CHER arms which illustrated that absolute CD4 counts were directly related to thymic output adjusted by age, i.e. as thymic output increases so does the CD4 count. This finding highlights the importance of preserving thymic output in children to optimize their potential for CD4 reconstitution and long-term immunological health.

5.3.3 Unstable HIV-infection and thymic output in CHER

The endpoint case-control comparisons demonstrated a trend towards lower thymic output in the “cases” at enrolment and prior to reaching the trial-defined endpoint. Lower thymic output in the “cases” prior to the endpoint is reinforced by the observations in the individual trajectories of lower or falling thymic output at the time of the endpoint, thereby inferring that poor thymic output may be associated with HIV progression.

ART-Def “cases” had significantly lower thymic output prior to the endpoint compared to ART-Def “controls”, whereas no significant difference was found between the “cases” and “controls” in the early-ART arms, suggesting that early ART may preserve thymic output. The paired measures of thymic output at 96 and 248 weeks in children that had reached an endpoint demonstrated the greatest increase in thymic output from all the available paired specimens. This is reflective of the findings that ART preserves and may even augment thymic output as seen in ART-96W at 7-8 years compared to healthy controls, as reported elsewhere [66].

The pneumonia case-control analysis demonstrated lower thymic output before and after pneumonia compared to healthy CHER controls without pneumonia. This is consistent with the theory that lack of TCR diversity from poor thymic output predisposes individuals to opportunistic infections. Even despite adequate CD4 counts, restricted TCR repertoires have been reported in HIV-infection [55] and may indicate that the CD4 T-cell pool has expanded from a limited number of clonotypes. Accelerated T-cell destruction in uncontrolled HIV is likely to restrict the TCR repertoire but T-cell generation dysfunction, such as impaired thymic output must also be present.

A selection bias exists in the case-control analyses since not all specimens from those that died were available. The analyses are also constrained by the fact that insufficient numbers of samples were available to adequately compare the groups considering the wide variation of thymic output and the power calculations performed; this was particularly true of the pneumonia analysis, where the numbers within this comparison were too small to detect any difference between early and deferred ART. However in view of the nature of both these limitations any differences detected here are likely to be underestimated.

Thymic Output in Paediatric HIV Summary

In summary, the CHER ART-strategies served most children fairly well in terms of preserving thymic output, however early ART for longer (i.e. ART-96W) appeared to sustain thymic output to that of a healthy child and be more beneficial long-term over deferred ART and shorter early ART. These data suggest thymic output temporarily increases as a consequence of untreated HIV-infection in the first months of life, plausibly due to a feedback mechanism from CD4 T-cell death plus antigen-stimulated naïve to memory T-cell conversion. This increase is unsustainable and results in depleted thymic activity as reflected by subsequent falling thymic output in the first months of life until ART is initiated. Augmented thymic output was demonstrated following ART-initiation either at enrolment, after interruption or as indicated in the deferred arm. Observations of low thymic output at the trial-defined endpoints, alongside the case-control and pneumonia analyses suggest low thymic output is associated with poor clinical outcome and may be due to limited functionality from a restricted TCR repertoire.

Data from the previous chapter showed a declining CD4/CD8 ratio and increasing levels of CD8 and CD8 activation despite HIV RNA viral suppression and on-going ART. This could suggest immune activation persists and more needs to be done to restore immunological health to that of an HIV-uninfected child. The immunopathogenesis of HIV is largely due to accelerated destruction combined with impaired generation of CD4 T-cells; while ART successfully reduces CD4 T-cell destruction and timely ART-initiation may prevent impairment of thymic output, there is a dearth of adjunct strategies to repair thymic damage and optimise immunity. Detailed analyses are needed to unravel the association between thymic output and various immune parameters and thereafter ascertain clinical approaches to facilitate the function of organs that generate naïve T-cells, such as the bone marrow, thymus, and mucosal immune system.

While these novel insights develop understanding of the influence of HIV and ART on the output of the thymus, further long-term studies are required to establish the relationship between thymic output and clinical health, and ultimately to advance ART-strategies to optimise the immunological health of HIV-infected children.

Chapter 6

Naïve B-cell output in healthy and HIV-infected children

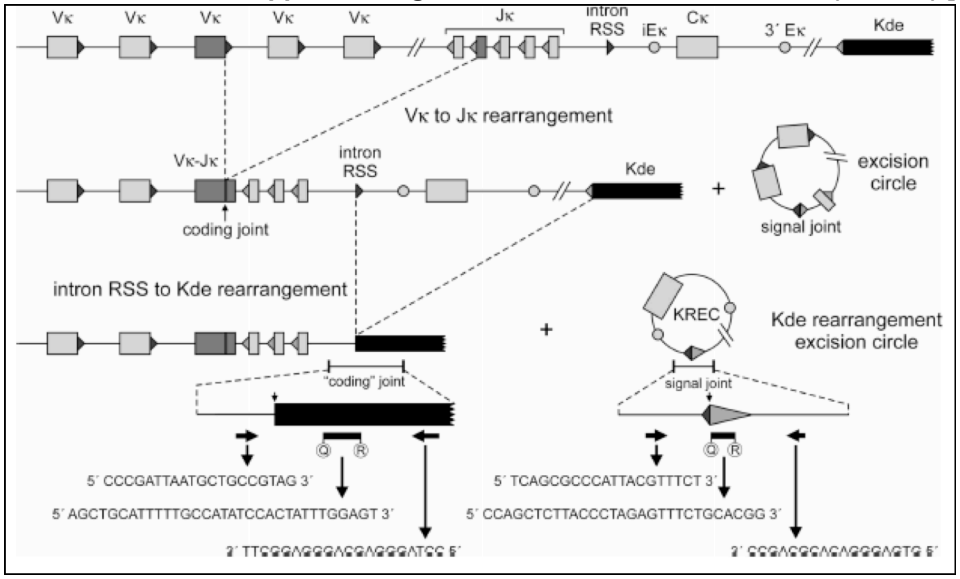
6.1 Background

While thymic output may be a crucial component for T-cell immune reconstitution in HIV-infected children, little is known about the role of naïve B-cell output in B-cell immune recovery and preservation, including the association with thymic output. There is no recognized approach to quantifying naïve B-cell output, although Kappa-deleting recombination excision circles (KRECs) are increasingly being used as a surrogate marker of bone-marrow output/activity. KRECs are stable double-stranded circular DNA structures, a product of variable (V), diversity (D), and joining (J) gene-recombination (Figure 6.1 [87]) that occurs during B-cell development as precursor B-cells mature into functional naïve B-cells. These multiple rounds of gene rearrangements generate a diverse range of functional B-cell receptors [2] and therefore the ability to respond to a wide range of pathogens.

KRECs are currently being used in some European countries and the USA for routine screening for primary immunodeficiencies characterized by the absence of T- or B-cells such as severe combined immunodeficiency (SCID), common variable immunodeficiency (CVID) and X-linked agammaglobulinaemia (XLA) [185]. There have been a small number of studies using quantification of KRECs to improve characterization of primary immunodeficiencies and monitor treatment such as

enzyme replacement therapy for SCID[2] and recovery from stem cell transplantation, however there is limited reference range data for KREC quantities in healthy children as a comparison.

Figure 6.1: Formation of kappa-deleting recombination excision circles (KRECs) [87].



KRECs are remnants of DNA that have been excised during gene rearrangement. They remain within the nucleus of one of the two daughter cells following cell division.

In HIV-infected adults, a correlation has been described between increased duration of untreated HIV-infection prior to commencing ART and reduced measures of KRECs [33], potentially reflecting suppressed bone marrow output. There are limited studies exploring B-cell dysfunction in HIV-infected children and no studies to date quantifying KRECs or estimating naïve B-cell output in this population.

The effect of HIV on naïve B-cell output could possibly be associated with the variable rates and decline of protective antibody concentrations in HIV-infected children. Antigen-specific immunoglobulin G as low as 5% GMC at a median of 6 years have been reported for measles [186], 43% at 1 year for Hib [187], 38% at 8 months for tetanus [188], and 48% at 5 years for the pneumococcal conjugate vaccine [189]. A better understanding of the relationship between naïve B-cell output and antibody responses to routine vaccinations might help optimize primary and booster vaccination schedules in HIV-infected children.

The absolute number of naive B-cells is theoretically regulated by four processes: output from the bone marrow, rate of survival in the periphery, loss of naive B-cells from naïve-to-memory switch after encountering antigen, and antigen-independent homeostatic proliferation. In view of this interplay, absolute measures of KRECs are

unlikely to provide a reliable estimate of naïve B-cell output, especially since KRECs, like TRECs, do not divide on proliferation of the cell in which they reside and they are consequently diluted upon cell division in the peripheral circulation. Therefore the thymic output formula [11, 82] has been adapted for this purpose combining quantification of KRECs with peripheral measures of naïve B-cells and rates of proliferation (Methods section 2.4.5).

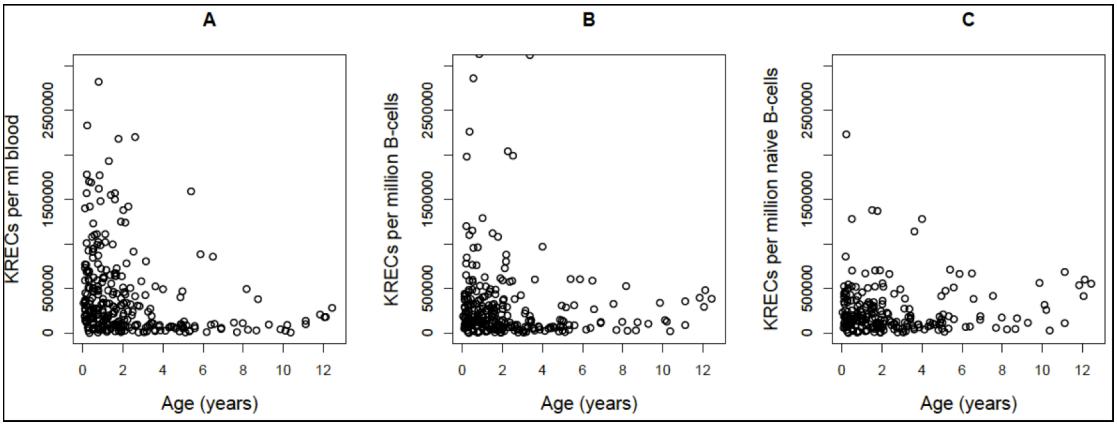
In this chapter, the normal ranges for measures of KRECs have been calculated and naïve B-cell output has been estimated in healthy South African children from birth to 13 years. To help understand the clinical significance of naïve B-cell output, the differences between naïve B-cell output in HIV-infected and uninfected children have been examined, along with the relationship with a range of antibody responses to routine childhood vaccinations and whether the ART-strategies used by CHER had an impact upon naïve B-cell output. Using data from the previous chapter, the relationship between thymic output and naïve B-cell output in both healthy South African children and HIV-infected children from CHER has also been explored.

6.2 Results

6.2.1 KRECs and naïve B-cell output in South African children

KRECs were measured from 288 cryopreserved PBMCs from the children of the CWC aged from 2 weeks to 13 years. Since there are currently no existing reference ranges for KRECs or naïve B-cell output, Table 6.1 explores five different ways of representing the reference ranges of these data: 1) KRECs per PBMC, 2) KRECs per mililtre of blood, 3) KRECs per million B-cells (CD19+ lymphocytes), 4) KRECs per million naïve B-cells (CD19+IgD+CD27- lymphocytes), and 5) using the adapted thymic output formula to estimate naïve B-cell output from the bone-marrow in naïve B-cells cells/day. The first two formats are practical and easy to calculate using output from the DNA PCR and basic parameters of the blood sample itself. The following two require flowcytometric measures of B-cells and naïve B-cells to give an estimate of the probable KREC quantity within these cells. However, only the fifth format accounts for the peripheral proliferation of naïve B-cells (Ki67+ proportion of CD19+IgD+CD27- lymphocytes) and is therefore likely to be more accurate, especially in cases of HIV-infection. Figure 6.2 presents the distribution of three of these measurements with age.

Figure 6.2: Possible representations of KREC data from healthy South African children.



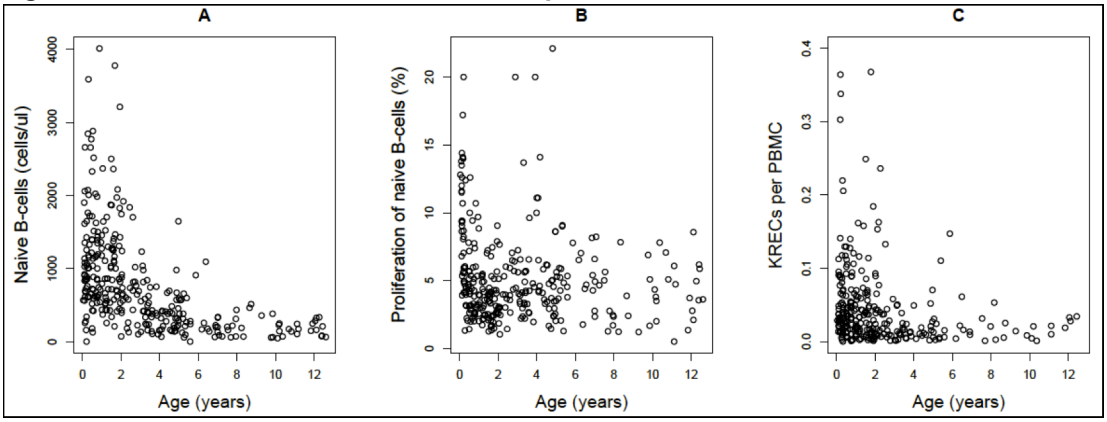
A) KRECs/ml blood. B) KRECs per million B-cells. C) KRECs per million naive B-cells. n=288. All y-axes limited to $3x10^6$ to visualize data omitting 4, 1 and 2 data-points from each respective plot.

Table 6.1: Reference ranges for KRECs and naïve B-cell output from the CWC.

Age-group	n	KRECs per PBMC	KRECs per mililtre of blood	KRECs per million B-cells	KRECs per million naïve B-cells	Naïve B-cell output (cells/day)
		Median, [IQR], (5^{th}–95^{th} centiles)				
0 – 3 months	33	0.03 [0.02-0.09] (0.01-0.32)	$3.5x10^5$ [2-$7x10^5$] ($1.2x10^5$ – $1.7x10^6$)	$2.2x10^5$ [1-$4x10^5$] ($5.6x10^5$ – $1x10^6$)	$2.1x10^5$ [1-$4x10^5$] ($6x10^4$ – $7.9x10^5$)	$3.2x10^8$ [1.9-$8.3x10^8$] ($2.2x10^7$ – $1.6x10^9$)
3 – 6 months	33	0.05 [0.02-0.09] (0.005-0.21)	$3.9x10^5$ [1.7-$6.9x10^5$] ($3.3x10^4$ – $1.7x10^6$)	$2.1x10^5$ [1.1-$3.8x10^5$] ($2.8x10^4$ – $1.1x10^6$)	$2x10^5$ [1.1-$3.7x10^5$] ($1.1x10^4$ – $5.5x10^5$)	$7.4x10^8$ [$2x10^8$-$3.7x10^9$] ($2x10^7$-$1x10^{10}$)
6 -12 months	50	0.04 [0.02-0.06] (0.002-0.12)	$3.4x10^5$ [1.7-$8.5x10^5$] ($4x10^4$ – $1.6x10^6$)	$1.6x10^5$ [1.2-$3.3x10^5$] ($1.4x10^4$ – $9.6x10^5$)	$1.7x10^5$ [1-$2.3x10^5$] ($1.2x10^4$ – $4.7x10^5$)	$6.6x10^8$ [$1.5x10^8$-$2.1x10^9$] ($2.6x10^7$-$6.8x10^{10}$)
1 -2 years	68	0.03 [0.01-0.06] (0.003-0.16)	$2.8x10^5$ [$9.7x10^4$-$6.4x10^5$] ($2.4x10^4$-$1.6x10^6$)	$1.4 x10^5$ [$7.4x10^4$-$2.9x10^5$] ($1x10^4$-$6.2x10^5$)	$1.5 x10^5$ [$7.2x10^4$-$3x10^5$] ($1.1x10^4$-$7x10^5$)	$4x10^8$ [$1x10^{8-}$ $2.3x10^9$] ($2.4x10^7$-$1x10^{10}$)
2 – 6 years	80	0.02 [0.009-0.04] (0.002-0.15)	$1.1x10^5$ [$6.1x10^4$-$4.2x10^5$] ($1.5x10^4$-$1.4x10^6$)	$8.9x10^4$ [$5.5x10^4$-$3.1x10^5$] ($1.2x10^4$-$1.1x10^6$)	$1.2x10^5$ [$7x10^4$-$2.7x10^5$] ($1.7x10^4$-$9.3x10^6$)	$1.4x10^8$ [$4.9x10^7$-$4.3x10^8$] ($3.8x10^6$-$4.4x10^9$)
6-12 years	24	0.02 [0.008-0.03] (0.001-0.05)	$9.7x10^4$ [$4.1x10^4$-$1.8x10^5$] ($9.9x10^3$-$4.8x10^5$)	$1.3x10^5$ [$8.1x10^4$-$3.4x10^5$] ($2.6x10^4$-$5.2x10^5$)	$1.9x10^5$ [$9.2x10^4$-$4.8x10^5$] ($4.2x10^4$-$16.6x10^5$)	$9x10^7$ [$1.3x10^7$-$1.7x10^8$] ($9.4x10^5$-$6x10^8$)

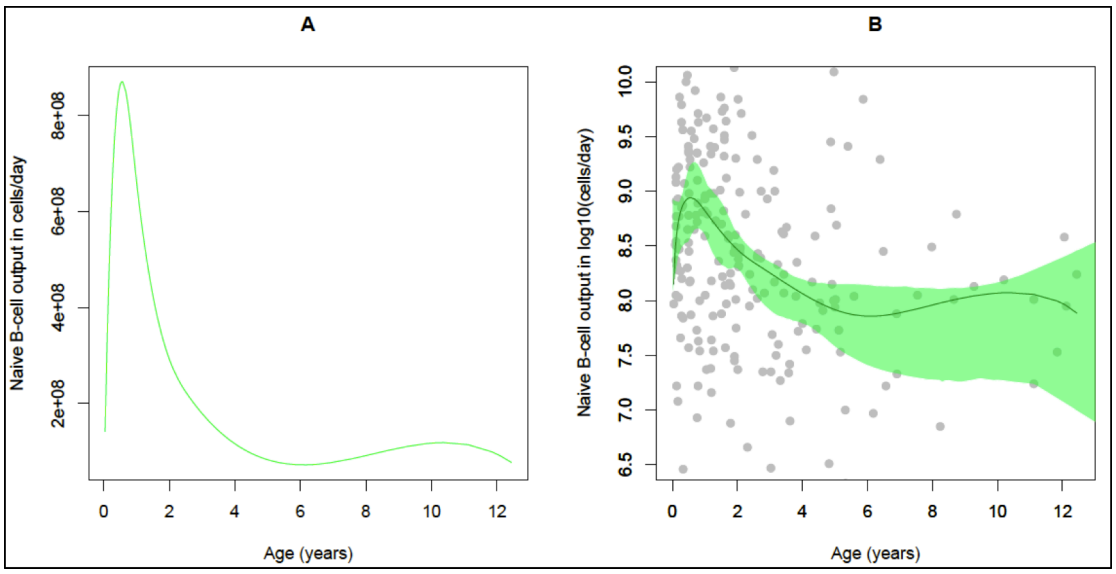
Naïve B-cell output has been used in most of the analyses done in this chapter, since the formula compensates for the peripheral proliferation that dilutes KRECS, thereby providing an explicit quantity of naïve B-cells entering the circulation. The elements of the naïve B-cell output model: quantity of naïve B-cells, proliferation of naïve B-cells and KRECs per PBMCs are represented in Figure 6.3 with the combined data plotted in a regression curve in Figure 6.4. The naïve B-cell output regression curve exhibits a steep increase and peak in the first year of life (median $8x10^8$ naïve B-cells/day), followed by a rapid decline to 6 years of age (median $7.2x10^7$ naïve B-cells/day) and subsequent relative plateau. The confidence interval of the median regression curve widens with increasing age due to less available data.

Figure 6.3: Elements of the naïve B-cell output model.



Plot A: quantity of naïve B-cells (CD3-CD19+IgD+CD27-). Plot B: proliferation of naïve B-cells (CD3-CD19+IgD+CD27-Ki67+ as a percentage of all naïve B-cells). Plot C: KRECs per PBMC (NB. Plot C y-axis limited from 1.0 to 0.4 omitting 4 data-points to visualize the distribution).

Figure 6.4: Naïve B-cell output regression curves across age from healthy South African children.

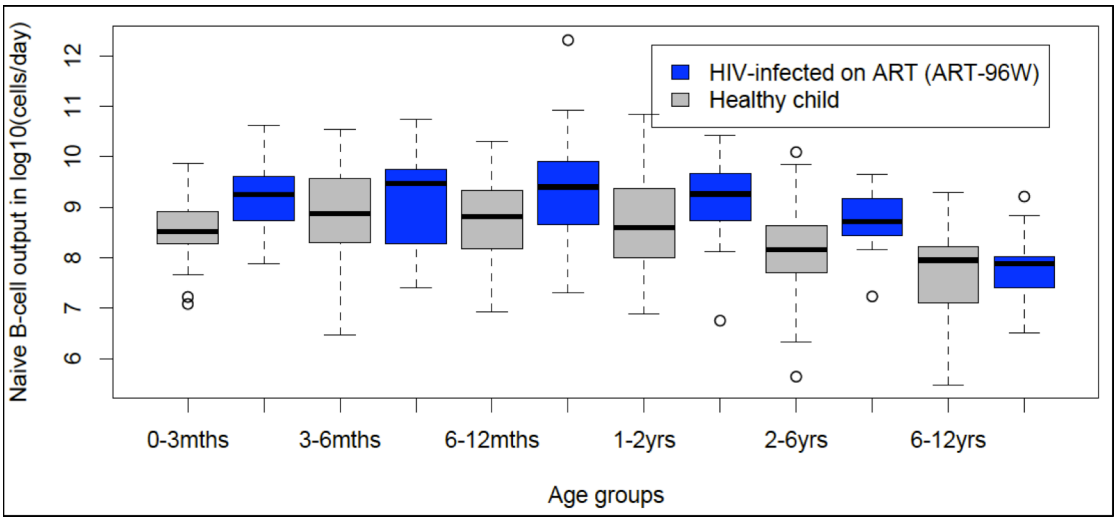


A) Regression curve representing naïve B-cell output in cells per day. B) Logarithmic curve with 95% CIs surrounding the centrally fitted line and gray circles representing each data point (n=288).

6.2.2 Naïve B-cell output in HIV-infected children

285 measures of naïve B-cell output were calculated from all 3 arms of the CHER trial from enrolment to CHER PLUS (7 weeks to 8 years of age). Measurements from ART-96W only were used for the purpose of exploring the effect of HIV and early ART on naïve B-cell output compared to healthy HIV-uninfected South African children from the CWC. Figure 6.5 illustrates children from ART-96W (n=139) had higher naïve B-cell output than their HIV-uninfected counterparts overall (Welch's t-test p<0.0001), particularly at 0-3 months of age (p<0.0001), 6-12 months (p=0.03) and 1-2 years (p=0.01). Figure 6.6 reinforces these differences with the data represented in the form of regression curves and Figure 6.7 demonstrates individual trajectories of naïve B-cell output that correspond to the overall regression curve.

Figure 6.5: Differences between naïve B-cell output in healthy HIV-uninfected children.



CWC (gray) and ART-96W (blue). 0-3 months: CWC n=33 vs. ART-96W n=34 (Welch's t-test p<0.0001); 3-6 months: 33 vs. 16 respectively (p=0.44); 6-12 months: 50 vs. 25 (p=0.03); 1-2 years: 68 vs. 29 (p=0.01); 2-6 years: 80 vs. 7 (p=0.14); 6-12 years: 24 vs. 28 (p=0.40). ART-96W initiated ART at median 7 weeks until 2 years, interrupting ART for a median 89 weeks in these children (median of 70 weeks overall for all of ART-96W).

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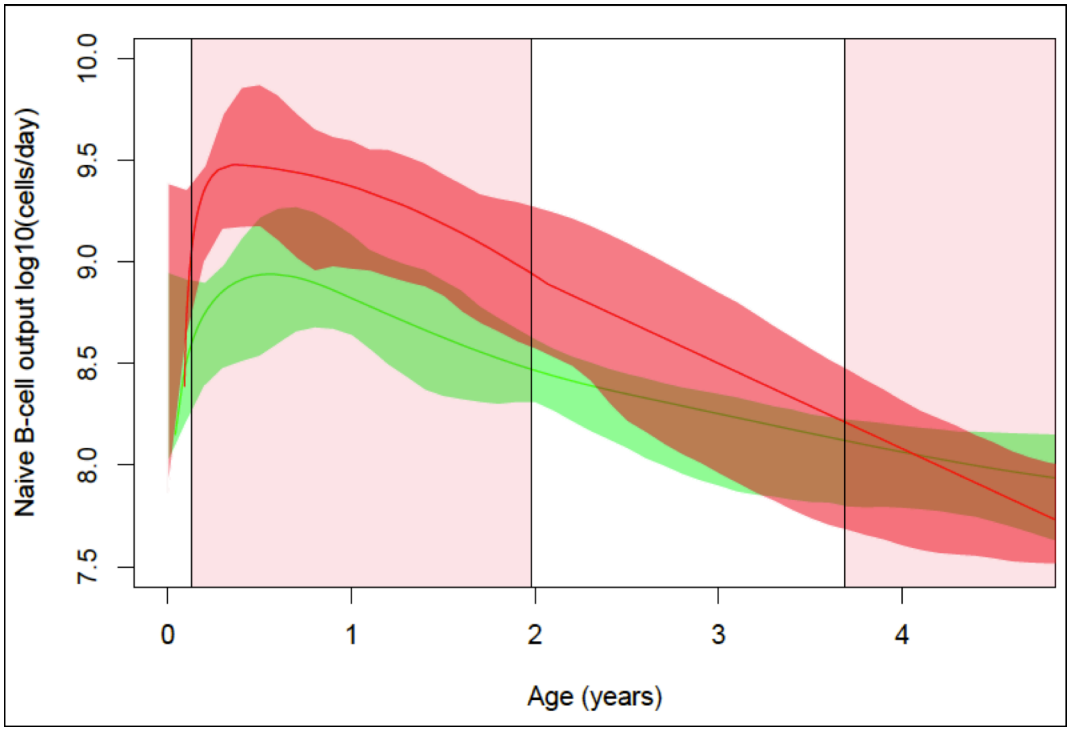
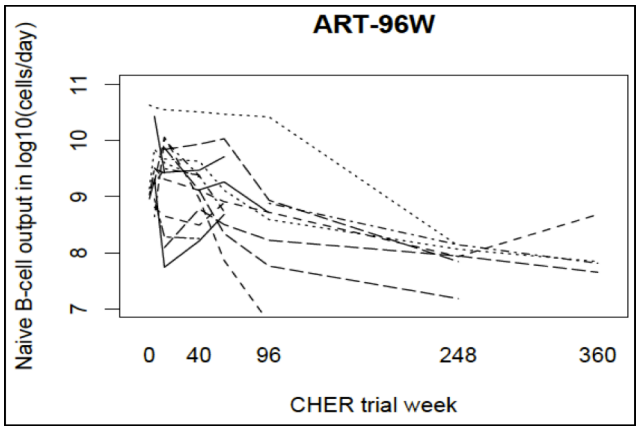
Figure 6.6: Naïve B-cell output regression curves of HIV-uninfected children compared to HIV-infected children until 5 years of age.



ART-96W is shown in red, n=111 samples in 70 individuals. ART-96W initiated ART at median 7 weeks until 2 years, interrupting ART for a median 89 (IQR 17-145) weeks in these 70 children. The central line is the best fit of the data and surrounding shaded areas are 95% CIs of the regression curve.

Figure 6.7: Individual trajectories of 17 children from ART-96W with at least 3 measures of naïve B-cell output from enrolment to 360 weeks follow-up.

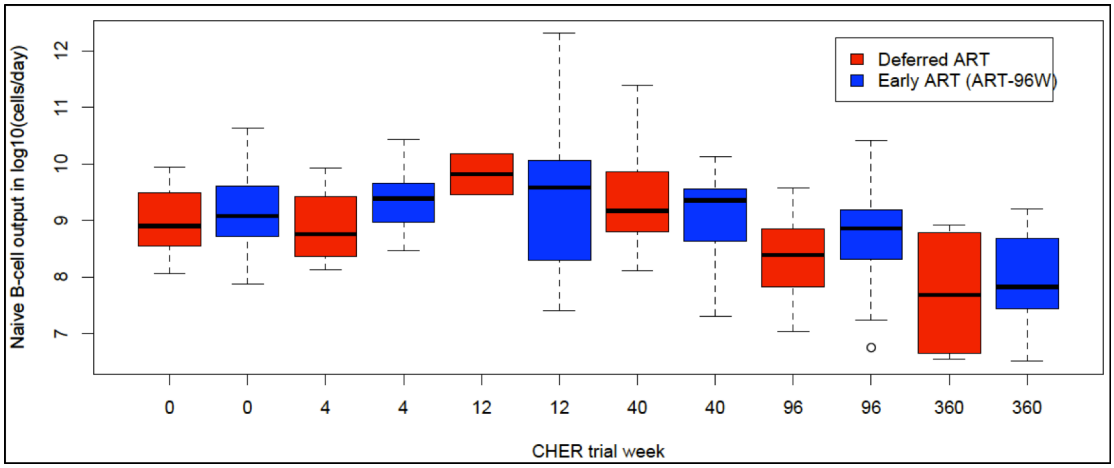


Impact of CHER ART-strategies on naïve B-cell output:

A) Deferred ART versus early ART

There was no significant difference in naïve B-cell output between deferred (ART-Def) and early ART (ART-96W) at all available paired time-points, although there was a trend towards lower naïve B-cell output in ART-Def in the first 4 weeks of the trial (Figure 6.8).

Figure 6.8: Naïve B-cell output in Log10(cells/day) in deferred ART (ART-Def, red) versus early ART (ART-96W, blue).

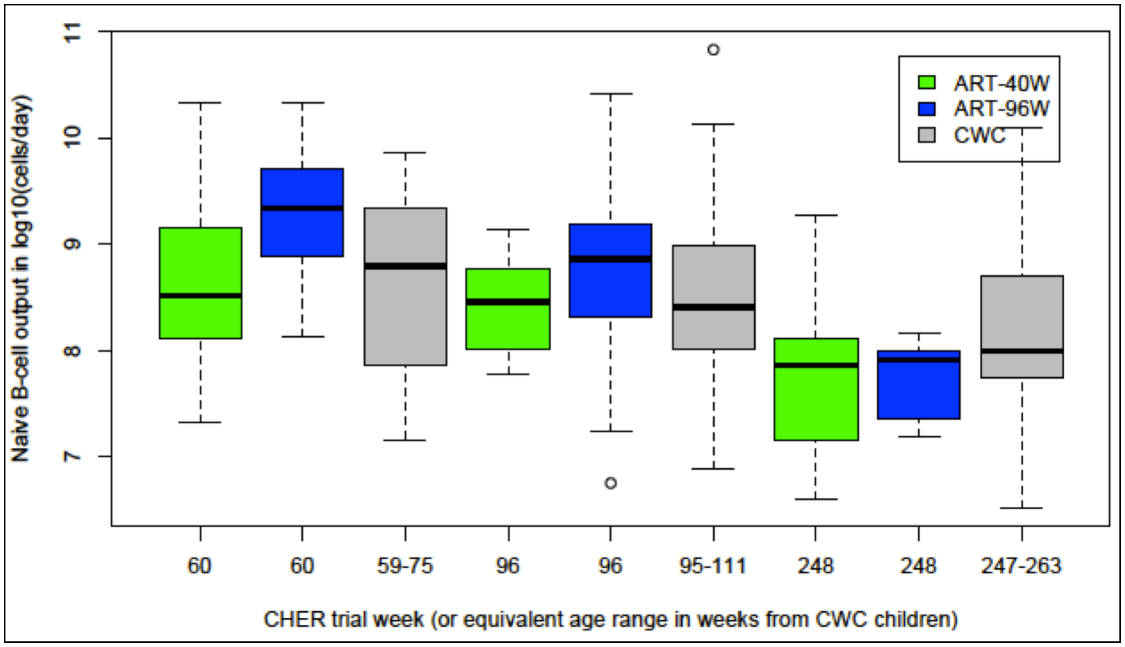


In these children ART-Def, in red, commenced ART at median 27 weeks of age and stayed on continuous ART. ART-96W, in blue, initiated ART at median 7 weeks until approximately 2 years of age, then interrupting ART for a median of 89 (IQR 17-145) weeks. (Numbers in each age-group were: ART-Def n=10 vs. ART-96W n=18 at enrolment; then respectively 4 vs. 17 at 4 weeks; 2 vs. 22 at 12 weeks; 26 vs. 19 at 40 weeks; 16 vs. 15 at 96 weeks; and n=9 vs. 10 at 360 weeks). No significant difference was identified between the arms compared at each time-point.

B) ART-interruption in the second or third year of life

ART-96W at 60 weeks had significantly higher naïve B-cell output over ART-40W (Welch's t-test p=0.004, Figure 6.9). At 60 weeks ART-96W were receiving continuous ART, however ART-40W had interrupted ART at 40 weeks, although by 60 weeks 71% of these samples were from children already back on ART. No further significant differences were seen at 96 weeks and 248 weeks, with 74% and 71% back on ART respectively in ART-40W, and 50% back on ART at 248 weeks in ART-96W. There was no difference in naïve B-cell output between those on ART or still off ART at 248 weeks in ART-40W (n=15 vs. 6, p=0.3), however in ART-96W those off ART at 248 weeks had significantly higher naïve B-cell output (n=9 vs. 9, p=0.02). There were no significant differences identified when either ART-40W or ART-96W were compared with HIV-uninfected children from the CWC.

Figure 6.9: The effect of ART-interruption on naïve B-cell output.



Both ART-40W and ART-96W started ART at median 7 weeks, and either interrupted ART in the second year of life after 40 weeks of ART (ART-40W, green) or in the third year of life after 96 weeks of ART (ART-96W, blue). Approximate age-matched healthy HIV-uninfected children are represented in gray for comparison. A significant difference was seen between the groups at 60 weeks (ANOVA p=0.006) but not subsequently. The number of children's specimens examined were as follows: at 60 weeks ART-40W n=21 vs. ART-96W n=21 vs. CWC n=17; at 96 weeks n=23 vs. 15 vs. 22; at 248 weeks n=21 vs. 18 vs. 14 respectively.

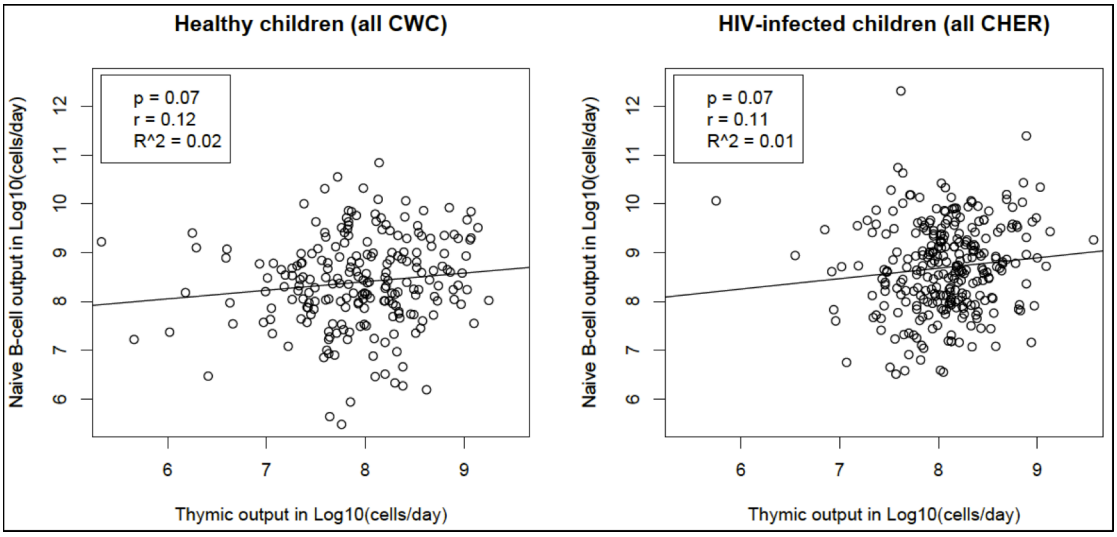
C) Overall long-term effect of CHER strategies

At 7-8 years of age there was no difference in naïve B-cell output detected between the 3 trial arms (ART-Def n=9, ART-40W n=14, ART-96W n=10, ANOVA p=0.52), and no difference compared to age-matched HIV-uninfected healthy children (n=23).

6.2.3 Naïve B-cell output and thymic output

There is a trend towards a significant correlation between naïve B-cell output and thymic output in healthy children (CWC cohort, Pearson's correlation p=0.07) and HIV infected children (all CHER arms combined, p=0.07) (Figure 6.10) although the spread of data is wide (R^2=0.02 and 0.01) and therefore the relationship is unlikely to be linear and directly related. There were no differences in the relationship between naïve B-cell output and thymic output in each of the CHER arms. Six CWC and 3 CHER children with both low naïve B-cell and thymic output were further investigated, however no association was identified with these combined low naïve cell outputs and any of the clinical or immunological data available.

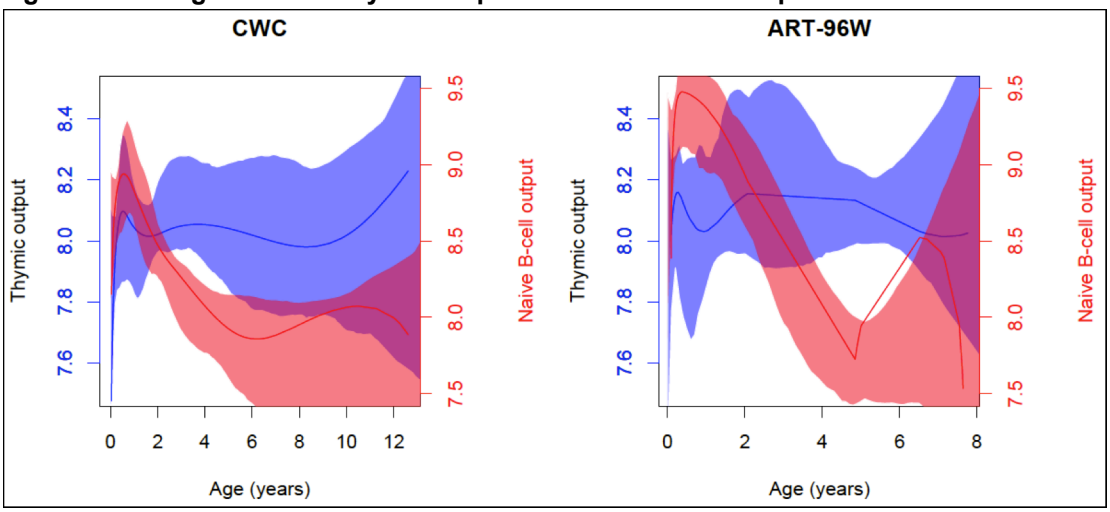
Figure 6.10: Relationship between naïve B-cell output and thymic output in healthy HIV-uninfected children and HIV-infected children from CHER.



The CHER specimens included here are from the combination of arms: ART-Def, ART-40W and ART-96W, on and off ART (n=285); CWC specimens n=288. Detailed analysis within arms and by ART status did not reveal any further association between thymic output and naïve B-cell output.

Examining the trajectories of thymic output and naïve B-cell output with age showed the peak thymic output corresponds to the peak naïve B-cell output in the first year of life in both CWC and ART-96W (Figure 6.11; NB. ART-96W was examined separately from the other arms of CHER so that the effect of the ART-strategies would not confound the overall impression). Both sets of children exhibit declining naïve B-cell output to 5-6 years of age while thymic output remains fairly constant. Following the trough of naïve B-cell output, both sets of children demonstrate a subsequent increase albeit greater in ART-96W. The median regression curve of ART-96W does not remain within the confidence interval calculated since there were no data between 5 and 6.5 years and only 10 data points between 6.5-8 years.

Figure 6.11: Regression of thymic output and naïve B-cell output.



Regression curves of thymic output (blue) and naïve B-cell output (red) in Log10(cells/day) with 95% confidence intervals of the line of best-fit. Healthy HIV-uninfected children (CWC, n=288) versus data from HIV-infected children on ART from 7 weeks of age until 96 weeks, restarting after a median of 38 (IQR 5-145) weeks ART-interruption (ART-96W, n=141 samples from 83 children).

Naïve B-cell output and functional antibody responses

The relationship between estimates of KRECs or naïve B-cell output was explored within individual CHER participants with a range of antibody responses measured after infant PCV7 and Hib vaccinations and again at 2 years of age (Methods section 2.2). Pearson's correlation was found to be significant with several serotypes in infancy and at 2 years of age, however on examining the data this relationship was skewed by one child with extremely high naive B-cell output (log 10.4 naive B-cells/day) and corresponding extremely high antibody responses. Removing this one child from the analysis left nothing significant whatsoever. In addition, no relationship was detected between naïve B-cell output and quantity of HIV-specific antibody (anti-gp120 IgG, methods described in section 2.4.6, results in Chapter 7) measured in CHER participants at 84 weeks of the trial, adjusted by age of ART-initiation ($r=0.09$, $p=0.6$). However, circulating B-cell count (CD19+ lymphocytes) and naïve B-cells (CD19+IgD+CD27- lymphocytes) were found to correlate with serum titres of antibodies against pneumococcal serotypes 4 and 18C (Pearson's correlations were all $p<0.004$, $r=0.5$).

6.3 Discussion

This chapter has been largely exploratory since little is known about normal KREC levels and the value of KREC measures in reflecting naïve B-cell output, in children and particularly in HIV-infected children.

The measures of KRECs and naïve B-cell output obtained from healthy HIV-uninfected children could form a reference dataset that might be useful in clinical practice for a number of paediatric conditions such as bone-marrow transplant, primary immunodeficiencies and potentially HIV-infection. Five different representations of the data have been proposed: KRECs/PBMC, KRECs/ml blood, KRECs/million B-cells, KRECs/million naïve B-cells and naïve B-cell output in cells/day. Although all measures reduce fairly rapidly after 2 years of age, the most biologically appropriate measure is probably the estimation of naïve B-cell output in cells/day. The naïve B-cell output formula accounts for the rate of naïve B-cell proliferation in the periphery, although the formula is limited since it assumes the mean KREC content of a naïve B-cell entering the peripheral circulation to be 0.6 as per the thymic output formula, and this is currently unknown and requires verification. However, each of the other representations do have their own value since because in the clinical setting it may not be practical to perform the intracellular immunophenotyping required to quantify Ki67 and thereby estimate naïve B-cell output. KRECs/ml of blood may somewhat overcome the bias of peripheral proliferation over using KRECs per unsorted PBMCs although would not be as accurate as naïve B-cell output. KRECs/million B-cells and KRECs/million naïve B-cells also have their value as the measure is localised to the cell subsets of origin and gives an idea of the proportion of new circulating naïve B-cells.

Not dissimilar to the predicted thymic output curve, the naïve B-cell output curve using KRECs and B-cell flowcytometry steeply increases from birth to peak around 1 year of age, followed by a rapid decline until 6 years and a lower peak between 6 and 12 years. The high naïve B-cell output coincides with the rapid development of the child's immune system during the first year of life. The subsequent rapid decline in naïve B-cell output may reflect the physiological activity of the bone marrow or may also be influenced by the trafficking of naïve B-cells to the lymphoid organs. The second smaller peak may be related to growth and hormonal changes leading up to puberty, or could be an anomaly in view of the fact that there was comparatively less data during this age (n=24) and the confidence interval appears far wider here.

The analysis of this chapter suggested there might be a positive correlation between naïve B-cell output and thymic output, perhaps reflecting progenitor cell development in the bone marrow of both T- and B-cell precursors; however the distribution of data is broad and it is likely that a multitude of factors are involved. Interestingly, it appears that naïve B-cell output is less variable than thymic output overall, although it is not clear whether this is a true finding or attributable to the stability of laboratory measures of TRECs, KRECs and flowcytometry on cryopreserved cells.

Naïve B-cell output may indirectly be increased with ART, as naïve B-cell output was overall significantly higher in HIV-infected children on ART compared to their uninfected counterparts for the first two years of life. This is reinforced by the decline of naïve B-cell output during ART-interruption after 96 weeks of ART, although further research is required to explore these important dynamics.

The numbers between groups comparing different ART strategies used by CHER were not always optimal especially between ART-Def and ART-96W. There was a trend towards lower naïve B-cell output in ART-Def, suggesting that longer time before commencing ART is likely to be inferior to early ART at least until 2 years of age. However, despite delayed commencement of ART, individuals in ART-Def, on continuous ART appeared to sustain the trajectory of naïve B-cell output to those who interrupted ART after 96 weeks. This pattern was also seen with lower naïve B-cell output in ART-40W at 60 weeks after they had interrupted for 20 weeks compared to ART-96W who had not yet interrupted, inferring that naïve B-cell output is being preserved by ART. The 3 different CHER strategies appear to have served the participants fairly in terms of preserving naïve B-cell output since by 5 and 7-8 years of age no significant differences were detectable between the trial arms.

In summary, this chapter has explored various representations of naïve B-cell output, the distribution in healthy HIV-uninfected children, the impact of the CHER ART-strategies upon naïve B-cell output and relationship with functional antibody responses. These analyses suggest that HIV-infected children on ART have higher naïve B-cell output than their uninfected counterparts, that deferred ART and ART-interruption are associated with lower naïve B-cell output compared to early or continuous ART, and that the relationship with thymic output or functional antibody responses are likely to be non-linear and multifactorial. However, any interpretation of these exploratory findings are tentative since there are numerous areas of further research required to validate these results, primarily to verify the assumptions made by the mathematical model.

Chapter 7

HIV-antibody seronegativity with early antiretroviral therapy

7.1 Background

In the absence of breastfeeding and early ART, infants acquire HIV either in-utero or at delivery and develop HIV-antibody responses by mean age 7.4 months [190]. Measurement of HIV-antibody is not recommended for HIV diagnosis below 18 months due to the persistence of maternal antibody [190, 191]; beyond 18 months HIV-antibody assays are used to diagnose and re-confirm HIV-infection. However, with early ART and effective viral suppression, there have been increasing case reports of infants not developing HIV-specific antibodies [47-51], probably due to reduced viral antigen exposure while maternal antibody is lost. Although rare, the decline of antibody titres has also been reported in HIV-infected children and adults on continuous ART initiated after infancy [192, 193].

The incidence of seronegativity in HIV-infected children is not known, and the management implications are potentially important as absence of detectable HIV-antibody could lead to misdiagnoses and inappropriate ART cessation. This is particularly relevant in many resource-limited settings where, for financial and logistical reasons, infants are started on ART following a single DNA PCR, and subsequent reconfirmation of HIV-status on ART using antibody tests then becomes

problematic. Since current ART guidelines recommend immediate ART for all HIV-infected children under 5 years [44], it is important that the frequency and key predictors of seronegative status in HIV-infected children receiving early ART are understood, so that antibody results may be correctly interpreted, especially where HIV DNA or RNA tests are not readily available. In addition, neither the short nor long-term clinical, immunological or virological consequences of seronegativity in HIV-infected children treated early in infancy are known. This maybe particularly relevant during subsequent planned or unplanned ART interruption where pronounced viral rebound after discontinuation of ART has been reported in HIV-infected seronegative children [49, 50].

Within a week of detectable HIV-viraemia in adults and children over 6 months of age, B-cell responses can be detected as antigen-antibody complexes [194] followed in days by circulating anti-gp41 antibodies and then anti-gp120 antibodies after several weeks [195]. These HIV binding antibodies are measured in standard diagnostic tests and, in contrast to neutralising antibodies, do not exert selective immune pressure on HIV [196]. Neutralising antibodies bind cell-free virus, preventing the virion from infecting host target cells, and thereby disrupting subsequent replication [195]. As HIV progresses the quantity of binding antibodies might infer the presence of neutralising antibodies [195]. B-cell depletion results in decline in both binding and neutralising antibodies, and a subsequent rise in plasma viral load rise [32], however in the context of ART initiated in early infancy HIV-seronegativity does not reflect B-cell depletion since viraemia is typically controlled with effective ART, nor should it reflect viral eradication [192], instead it is likely to be simply due to lack of antigenic stimulation.

This chapter describes the frequency of HIV-seronegativity by comparing antibody results taken from children who started early ART compared with deferred ART in the CHER trial. This work has been published in Lancet Infectious Diseases (Appendix XVII, [197]). The methods are described in section 2.4.6.

7.2 Results

7.2.1 HIV-antibody in early versus deferred ART

One hundred and nine plasma samples were available from 143 children randomised to ART-96W and 75 samples from 125 children in ART-Def. 34 ART-96W and 50 ART-Def samples were not tested because: plasma not stored (10 vs. 11) or used for other reasons (1 vs. 10), children lost to follow-up (11 vs. 6), withdrew from study (1 vs. 1) or died prior to trial week 84 (11 vs. 22). The following numbers of specimens were processed for each HIV antibody assay: anti-gp120 ELISA (109 ART-96W vs. 75 ART-Def), automated serology (107 vs. 75), and rapid antibody test (101 vs. 74) due to volume of sample available.

There were no significant differences between ART-96W and ART-Def children in gender, ethnicity, CD4 count/percentage or viral load at enrolment or week 84 (Table 7.1). Most children in the CHER trial were formula-fed, with no difference between treatment groups (ART-Def 67 [89%] of 75; ART-96W 100 [92%] of 109). As per trial design, a significant difference exists in median age at ART initiation between ART-96W and ART-Def, median 7 (7-8) weeks versus (23 (IQR 18-32) weeks respectively). Of all 184 children's samples analysed, 94% had received at least 24 weeks of ART by trial week 84. In ART-Def 67/75 (89%) had commenced ART by week 84. Of the 8 children who were not on ART, 4 started aged 3-5 years and 4 remained off ART; all 8 were seropositive.

Children in ART-96W were significantly more likely to be seronegative than ART-Def by the two commercial tests. 49 (46%) ART-96W versus 8 (11%) ART-Def were seronegative by automated serology ($p<0.0001$), and 54 (53%) versus 8 (11%) by rapid antibody test ($p<0.0001$, Table 7.2). Although most results were concordant between automated serology and the rapid test, 9 children in ART-96W with weakly reactive automated results had negative rapid tests. The 8 seronegative children from ART-Def started ART at median age 9.9 weeks (range 7.8-22.9), with median CD4 count 1521 cells/μl (range 939–2862) or CD4% 33 (31-43) and enrolment viral load at upper limit of detection (750,000 copies/ml).

Table 7.1: Characteristics of children whose plasma were analysed for HIV-antibody. *Difference in numbers of each group detailed in text. **Age of ART-initiation differed significantly between arms, as per CHER design.

	ART-Def	ART-96W
	n (%)	
No. samples	75* (41)	109* (59)
Gender male	33 (44)	48 (44)
Ethnicity Black [Remaining mixed race]	74 (99)	105 (96)
No. reached primary outcome in CHER trial	18 (24)	16 (15)
	Median [IQR]	
CD4 at enrolment: - Absolute count (cells/μl) - Percentage	2,039 [1,587 – 2,861] 34 [28 - 41]	2,067 [1,520 -2,743] 35 [30 - 39]
CD4 at 84 wks. - Abs count (cells/μl) - Percentage	1,623 [1,330 – 2,074] 34.2 [31 – 40]	1,641 [1,247 – 2,065] 36.9 [31.7 – 42.1]
Log10 viral load at enrolment	5.88 [5.68-5.88]	5.88 [5.72-5.88]
Percentage with viral load <400 copies/ml at 84 wks.	60 (80%)	91 (83.5%)
Log10 viral load at 84 wks of trial in unsuppressed	4.71 [3.78-5.16]	4.17 [4.01-4.79]
Age of starting ART (weeks)**	22.9 [18.2 – 31.2]	7.3 [6.8 – 8.3]

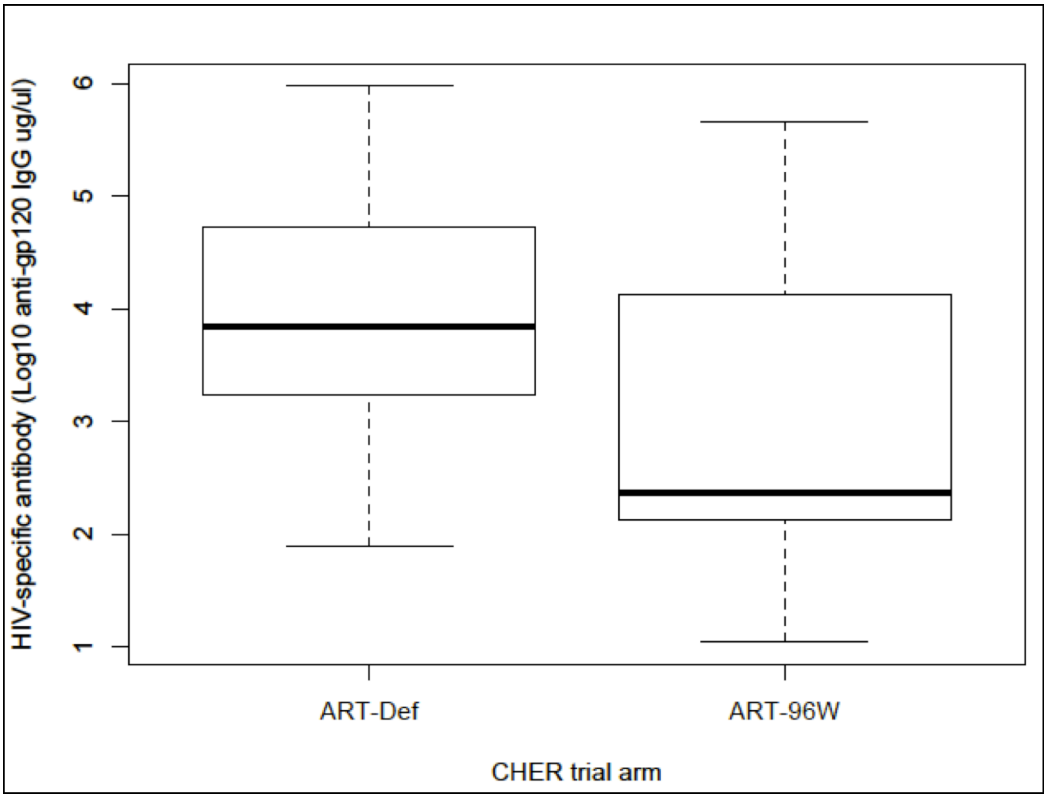
Table 7.2: Comparison of automated serology ("Serology") and rapid antibody test ("Rapid") between ART-Def versus ART-96W at CHER trial week 84.

	ART-Def n (%)		ART-96W n (%)		
	Serology	**Rapid**	**Serology**	**Rapid**	**p-value**
Total processed	**75**	**74**	**107**	**101**	
Reactive	62 (83)	64 (86)	38 (36)	41 (41)	
Weakly reactive	5 (7)	2 (3)	20 (19)	6 (6)	<0.0001*
Non-reactive	8 (11)	8 (11)	49 (46)	54 (53)	
Insufficient	0	1	2	8	
Sensitivity	**89.3%**	**89.1%**	**54%**	**56.5%**	

*Chi-square test for difference between CHER arms is p<0.0001 for both routine antibody test methods (serology and rapid antibody tests). Sensitivity of these antibody tests was calculated based on known HIV-infection in all CHER participants, diagnosed by HIV DNA PCR and confirmed with a RNA viral load >1000 copies/ml in all children at enrolment. A weakly reactive test will yield a weak line present in the test area and follow-up testing is recommended to confirm an initial weakly reactive result.

Total IgG levels were similar in both ART-96W and ART-Def (median 25,993 µg/µl [IQR 17,779-44,241] versus 25,530µg/µl [IQR 15,568-40,391], p=0.28). However, the quantitative anti-gp120 ELISA showed significant differences between ART-96W (median 230 µg/µl [IQR 133–13,129]) and ART-Def (6,870 µg/µl [IQR 1,706–53,645]), p=0.04, Figure 7.1). In all children, higher anti-gp120 IgG were strongly associated with seropositivity by automated serology with median 162µg/µl [IQR 102-223] in seronegative versus 9,984 µg/µl [630-70,469] in seropositive children, p<0.0001. The 8 children from ART-Def who were not on ART by week 84 demonstrated very high anti-gp120 IgG (median 336,687 µg/µl). No anti-gp120 IgG was detected in any of the 18 HIV-uninfected controls, whereas all samples from the 184 CHER participants had detectable anti-gp120 IgG.

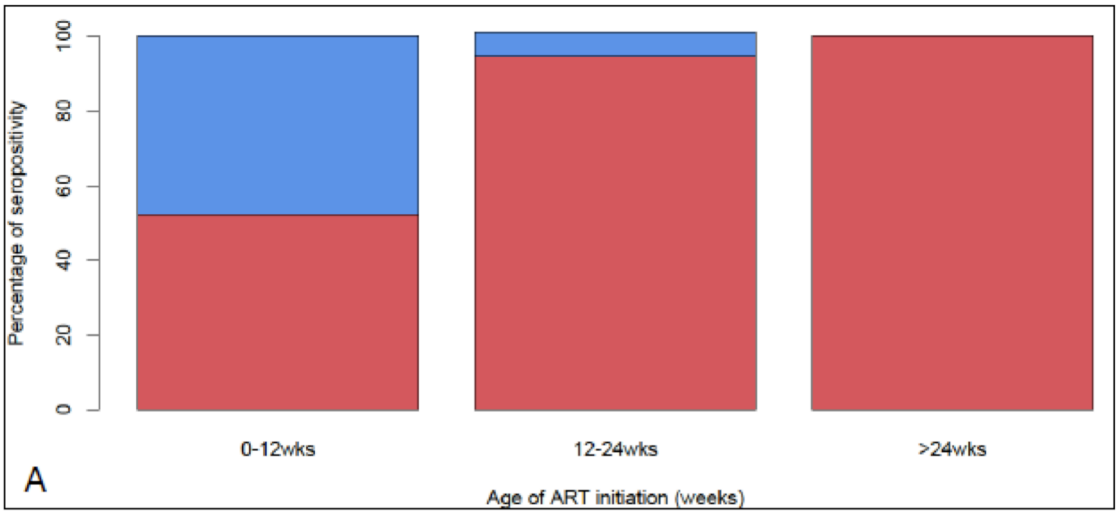
Figure 7.1: Distribution of HIV-specific antibody between deferred and early ART.



Distribution of anti-gp120 antibody by ELISA between ART-Def (n=75) and ART-96W (n=109) at CHER trial week 84 (p=0.04). Anti-gp120 IgG was detectable in all children.

By automated serology, starting ART between 12-24 weeks old had 13.7-fold higher odds for seropositivity at trial week 84, compared to starting ART aged 0-12 weeks (95% CI: 3.1–60.2, p=0.001). All 33 children starting ART after 24 weeks old were seropositive at week 84. Figure 7.2A presents frequency of HIV antibody seropositivity by automated serology at week 84 according to age at ART initation. The significant association between age at ART initiation and seropositivity also holds when the analysis is restricted to ART-96W only.

Figure 7.2: Frequency and probability of HIV antibody seropositivity.



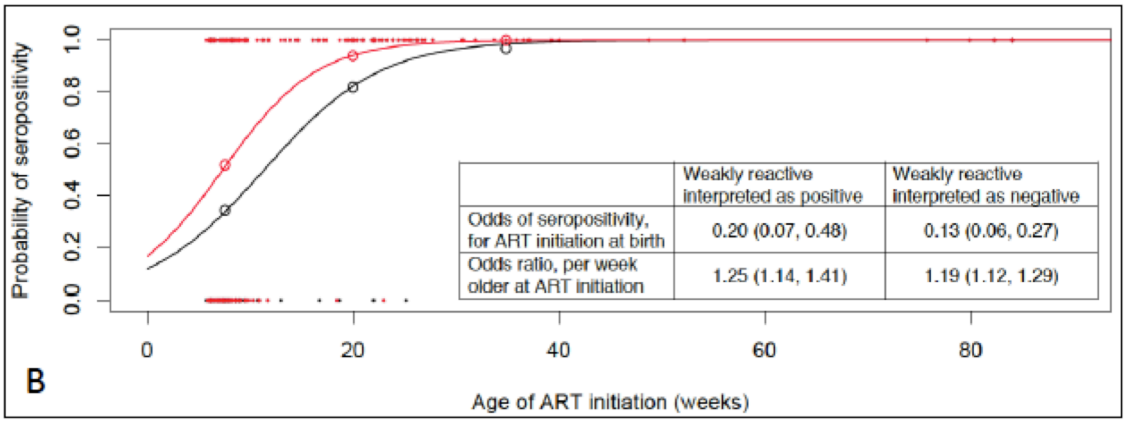


Figure 7.2A: Frequency of HIV antibody seropositivity by automated serology at 84 weeks of the CHER trial (~2 years of age) according to age of ART initation. The bar chart demonstrates the percentage of children who were seropositive at 2 years depending on whether commencing ART at 0-12, 12-24 or after 24 weeks of age (Blue = seronegative, Red = seropositive; here, a weakly reactive serology was interpreted as seropositive).

Figure 7.2B: Estimated probability of HIV seropositivity at ~2 years of age derived from a logistic regression model fitting antibody response on age at ART initiation as linear in a logit scale. Here, the red data represents where weakly reactive serology was interpreted as seropositive, and the black data represents weakly reactive serology being seronegative; the circles represent the proportion of seropositivity in equally-sized groups for age at ART initiation; individual data-points are represented by the red or black dots at 0.0 (seronegative) or 1.0 (seropositive) on the probability y-axis. The inset table gives the odds or odds ratio and 95% confidence intervals of the two logistic regression models.

Logistic regression analysis demonstrates a strong association between serostatus and age of ART initiation (OR 1.24 95% CI 1.12-1.38), which reflects a 24% increase in the odds of seropositivity for every week before ART is initiated (Figure 7.2B. The effect of viral load (estimated by area-under-the-curve viral load until week 84) is also

significant with OR 1.67 (95% CI 1.19-2.33), increasing the odds of seropositivity by 67% for every 100 copies of HIV RNA per militre blood. When the effect of viral load on seropositivity is adjusted for age of ART initiation, and vice-versa, both remain significant with age of ART initiation decreasing to OR 1.14 (95% CI 1.0-1.32), and viral load OR 1.59 (95% CI 1.1-2.3) (Table 7.3). CD4 count, and CD4 percentage when adjusted for viral load did not affect the odds of seropositivity. P-values for the null hypothesis that age at ART initiation doesn't affect the log-odds are highly significant in both models, where weakly reactive was interpreted as positive or negative (p<0.0001). In addition, older age at ART initiation was also associated with increased anti-gp120 IgG (Spearman's rank correlation p=0.002, coefficient=0.35).

Table 7.3: Univariate and adjusted logistic regression of serostatus against ART initiation age, viral load and CD4%.

	Univariate analysis			Adjusted analysis		
	OR	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
ART initiation age	1.24	1.1-1.4	<0.0001	1.15	1.0-1.3	0.06
AUC viral load	1.67	1.2-2.3	0.003	1.59	1.1-2.3	0.02
CD4%	1.0	0.9-1.0	0.04	1.0	0.9-1.1	0.71

7.2.2 HIV-antibody and HIV RNA viral load

In all children, cumulative log-viral load from study enrolment until week 84 correlated with anti-gp120 IgG levels (Spearman's rank correlation p<0.0001, coefficient=0.54) (Figure 7.3). Children with high viral exposure had high HIV-specific antibodies; however children with less cumulative viral exposure (i.e. <7.8 log) showed greater variation in HIV-specific antibody levels.

Neither serostatus nor quantity of anti-gp120 IgG appeared to correlate with subsequent time spent off ART after stopping at week 96 in ART-96W (median 54.9 weeks off ART in these 109 children [IQR 12.7 – 140], Spearman's rank correlation both p>0.26, coefficient=0.11). All 71/109 (65%) children who had viral load available during ART-interruption exhibited HIV viral rebound during interruption. However, examining the viral loads revealed a contrasting pattern of HIV RNA resurgence by serostatus during interruption (Figure 7.4). Although not statistically significant, seronegative children have a median 0.44-log higher HIV viral rebound than seropositive children at first viral load during ART-interruption; and subsequently seronegative children began to suppress their viral load by the second measurement, whereas seropositive children demonstrated an increasing viral load.

Figure 7.3: Relationship between quantitative anti-gp120 IgG and cumulative viral load from enrolment until trial week 84. Cumulative viral load was summarized by area under the time-versus-log(viral load) curve from randomization to week 84. Spearman's rank correlation p<0.0001, coefficient=0.54.

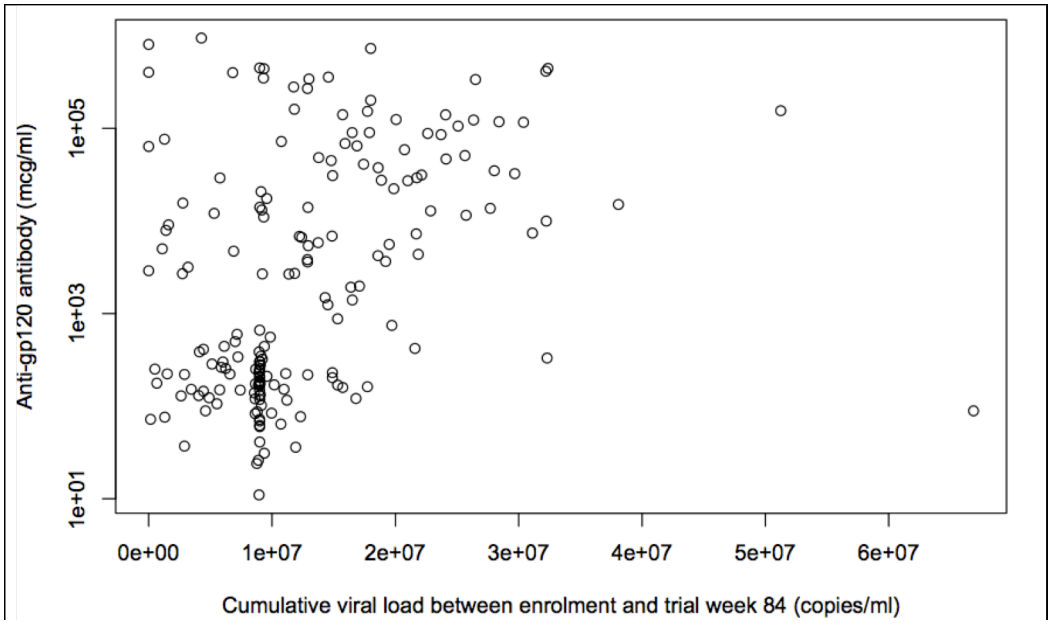
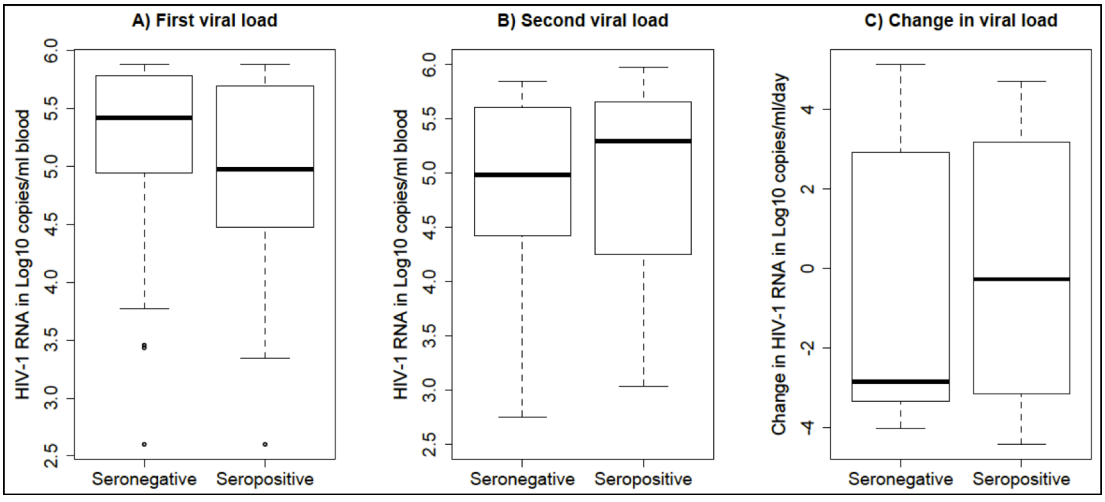


Figure 7.4: HIV RNA viral load resurgence after ART-interruption by serostatus.



A) First viral load after ART-interruption (median 8, IQR 8–20 weeks from interruption, n=72, t-test p=0.36). B) Second viral load after ART-interruption (median 20, IQR 20–34 weeks from interruption, n=54, t-test p=0.49). C) Change in viral load during ART-interruption from first to second available viral load, quantified in change of HIV RNA copies/ml per day (n=54, t-test=0.28).

7.3 Discussion

We have shown that nearly half of children starting ART in the first 12 weeks of life are seronegative for antibodies to HIV using commercial assays at approximately 2 years of age, compared with only 6% of infants starting ART aged 12-24 weeks and none starting ART thereafter. Furthermore, concentrations of antibodies to HIV gp120 were significantly correlated with HIV viral loads suggesting an important role of antigenic stimulation in antibody production.

Although HIV-seronegativity has been previously described [47-50], this is the first study to formally compare HIV antibody values taken at the same age from children who started early ART compared with deferred ART in a randomised trial. A key implication of our results is that in children with early diagnosis and treatment before 6 months, a negative antibody test is a frequent occurrence, particularly among those starting before 12 weeks. All ART-96W children experienced HIV-RNA rebound following ART interruption at week 96; thus, a negative antibody assay does not indicate absence of HIV.

For numerous reasons it may be necessary to reconfirm HIV-status in children on ART from early infancy. The accessibility, accuracy and false positive rates of HIV proviral DNA PCR in some resource-limited countries are far from ideal, especially with older assays [198], and children are started on ART without confirmation of HIV status [199]. There are anecdotal reports that caregivers not infrequently doubt the initial diagnosis and request confirmation from healthcare practitioners, or purchase over-the-counter rapid antibody tests, which are increasingly available. When faced with a negative HIV-antibody result it may be difficult to convince families to continue therapy, which may ultimately be to the detriment of the child.

In view of the limitations of present commercial antibody tests in this setting, WHO stresses the importance of repeating HIV DNA PCR in children just before ART is initiated, but not waiting for the results before ART is started. If discordant with the initial DNA PCR, a third tie-breaker DNA PCR would guide any decision about stopping ART. Where confirmation of status is requested in a child started on ART before 6 months old, DNA PCR should be repeated and HIV-antibody tests should not be used. If the DNA PCR result is negative and families insist on stopping ART, interruption should be undertaken with staggered stopping for non-nucleoside reverse-transcriptase inhibitors [200], and might be followed with antibody testing. Preliminary data from quantitative DNA PCR analysis in CHER show that even

children with negative DNA PCR after early ART have viral resurgence after stopping ART [51, 201].

In view of increased focus on early treatment strategies to limit reservoir size [202], more sensitive simple diagnostic tests are needed. Our data confirm previous studies showing that rapid HIV antibody tests are less sensitive than automated serology [203]. Considering all CHER participants tested had detectable anti-gp120 IgG, clinical tests using these envelope antigens might improve diagnostic accuracy alongside proviral DNA assays and be cheaper than viral load testing.

Analysis demonstrated that age of ART initiation and viral load are strong predictors of serostatus, and adjusted analyses demonstrate that they both have independent contributions. The association between cumulative plasma HIV RNA and anti-gp120 IgG levels suggest that antibody production is related to HIV antigen-stimulation. Whether persistence of anti-gp120 IgG represents slow decay or is a response to low levels of HIV replication, requires serial measurements alongside comprehensive analysis of cell-associated HIV DNA and RNA. Detecting low-level replication despite sustained virological control could support investigating potential novel targets for eradication strategies.

There was no correlation between levels of anti-gp120 IgG and duration of time tolerated off ART in ART-96W; however studies using quantitative measurements of DNA PCR alongside more detailed mathematical analysis of CD4 and viral load trajectories throughout ART-interruption in CHER are underway.

We cannot extrapolate our results to all children starting ART over 24 weeks old since the number of children starting ART after 6 months in CHER was small and they were likely to be symptomatic. Other limitations include the fact that children from ART-Def had fewer samples than ART-96W, due mainly to higher numbers of deaths. However, this difference might, if anything, increase the proportion of early treated children being seronegative. We also cannot comment about HIV-antibody status among breastfeeding children starting ART, as most children in CHER were formula-fed.

Due to the study design of CHER, the effect of age of ART-initiation is indistinguishable from duration of ART until trial week 84. However, in adults loss of antibody is not reported unless the individual was either receiving pharmacological immunosuppression or initiated ART rapidly after primary infection [204], therefore

this suggests that age of ART-initiation plays an important role in the suppression of HIV-antibody.

In addition to age and timing of ART-initiation, HIV-antibody status and quantity is likely to depend on timing of transmission, maternal virus inoculum size and strength of immunological responses. Although routine antibody tests to confirm HIV-status cannot be recommended in this population, the strong relationship between cumulative viral load and anti-gp120 IgG levels, support a possible role for antibody quantification to assess extent of virological control or reservoir size [205]. These assays might also be used to improve clinical management since high HIV-antibody quantity or seropositivity after early-ART could be a marker of poor adherence, inappropriate dose, drug absorbance/metabolism issues, or a combination of these factors.

In conclusion, this study demonstrates that almost half of the children starting ART within 12 weeks of birth and 6% of those starting at 12-24 weeks were seronegative by 2 years of age. Therefore, clinical results from automated serology and rapid antibody assays cannot be used as evidence for HIV-infection among these children. WHO is planning to update early infant diagnostic guidelines in 2015 (Penazatto M, WHO Paediatric HIV Department, personal communication) and should take account of our findings. Clear guidance is needed for children of caregivers seeking HIV-status confirmation of their children, along with monitoring strategies for children who interrupt ART because the caregiver questions the original diagnosis of HIV.

Chapter 8

HIV proviral DNA and early antiretroviral therapy

8.1 Background

The introduction of antiretroviral therapy (ART) early in life has effectively reduced morbidity and mortality [45, 68] and optimised CD4 cell reconstitution [206]. Although HIV virological suppression is achievable in most children, HIV remains latent and integrated within the host genome in subpopulations of infected cells [207]. This reservoir, commonly estimated by quantitative measures of HIV proviral DNA [208, 209], is found in many cell types including CD34 stem cells, central nervous system (CNS) macrophages, astrocytes and dendritic cells [210-212]; however CD4 memory cells are considered a critical reservoir due to their longevity, homeostatic cell division and potential for reactivation on antigen encounter [213].

In the context of sustained virological suppression, there may be two or three phases in decay of proviral DNA reflecting the different half-lives of various cell populations [62]. Although HIV proviral DNA decay kinetics appear to vary over time and between individuals [50, 214], in a study of 14 children aged 3-14 years the reservoir was found to decay with a median half-life of 5 months over the first 1-9 months of ART, decreasing to a half-life of 30 months thereafter [214]. HIV eradication on stable

continuous ART might therefore only be possible after more than a decade of ART. Adult studies have reported reduced latent reservoirs in patients commencing ART in the early course of infection compared to the chronic phase [215-217]. Similarly, paediatric studies are increasingly demonstrating the same importance of early ART-initiation in reducing reservoir formation [202, 218, 219]. Numbers in these studies however are small and data is largely descriptive since patients are typically from resource-rich settings and have heterogeneous ART histories and demographics.

There is increasing interest in viral reservoirs since reports of functional cures in adults treated soon after infection [204]. Functional cure is defined as lack of detectable virus in the blood with a functional immune system without the need for ART, even though HIV can still be detectable within cells with sensitive assays. The case report of the “Mississippi Baby” has focused attention on early ART followed by interruption in children. This infant was given ART from 31 hours of age and rapidly suppressed plasma HIV-RNA to below detectable limits. The mother discontinued ART 15-18 months of age and when retested at 23 months of age, the infant was well with undetectable plasma HIV-RNA on conventional testing. The infant maintained virological suppression for more than 2 years before viral resurgence [220, 221] and detailed studies revealed only traces of proviral DNA while off ART. Novel approaches to obtaining a functional cure are being pursued, including depletion of T-cell subsets known to have integrated HIV DNA, elimination of the latent reservoir through activation and clearance mechanisms, and processes that interfere with memory CD4 T-cell homeostasis [213, 222, 223]. As these are not presently considered viable options for either adults or children, early ART initiation remains the therapeutic focus [224].

Current WHO policy recommends ART for all HIV-infected infants up to 5 years of age regardless of CD4 percentage, viral load or CDC staging[44]. In the absence of robust clinical trial evidence to support these recommendations, European guidelines advise ART for all infants under 1 year and to treat children aged up to 3 years old if CDC stage B/C, CD4%<25% (or <1000 cells/ul) or viral load >10^5 copies/ml [225]. In children who have already survived the first year of life, initiation of ART before their CD4 count drops below the threshold recommended level by the WHO [226, 227] has been linked, using mathematical modeling techniques, to improved long-term CD4 outcome [206]. The same may be true for the decay of the proviral DNA reservoir [202], since reservoir size has been associated with time to first undetectable viral load while on ART [218], reinforcing the aim of rapid suppression of plasma viraemia after commencing ART in any child. In addition to early ART,

there may be other factors that could enhance reduction of levels of HIV proviral DNA such as maintaining stable CD4 counts [228] and reducing immune activation[229-231], therapeutic vaccinations [232] and treating co-infections such as cytomegalovirus (CMV) or Epstein Barr Virus (EBV) [233, 234].

It is not known whether the duration of initial therapy is critical in reducing the reservoir nor whether ART-interruption within early childhood years might be detrimental to reservoir clearance, or even functional for the developing immune system [50] by inducing development of HIV-specific antibody responses. ART interruption has its benefits of convenience, reduced costs and potential reduction in rates of drug-toxicity, side effects and resistance. Although adult studies such as the SMART trial have demonstrated significantly higher risk of disease progression with structured ART interruption [235], there is less evidence to show the same is true for ART-interruption in children. Studies in adult patients who started ART very early in their primary infection have shown safety and low disease progression [204, 236, 237]. Although not encouraged, ART-interruption in children has not been associated with adverse clinical, immunological or virological consequences [68, 238, 239] and may be an acceptable option for certain situations such as where adherence is an insurmountable issue.

Formation and stability of the HIV reservoir in the presence of ART is not well understood [240-242], particularly whether its persistence is primarily due to longevity of latently infected cells or to low-level replication [243-245]. Further knowledge in these areas could be valuable in establishing practical approaches towards functional cure in children from high disease-burdened settings. Even in the absence of a functional cure, the long-term impact of different ART-strategies on reservoir size is now of considerable interest with potential to inform future strategies on ART management in childhood.

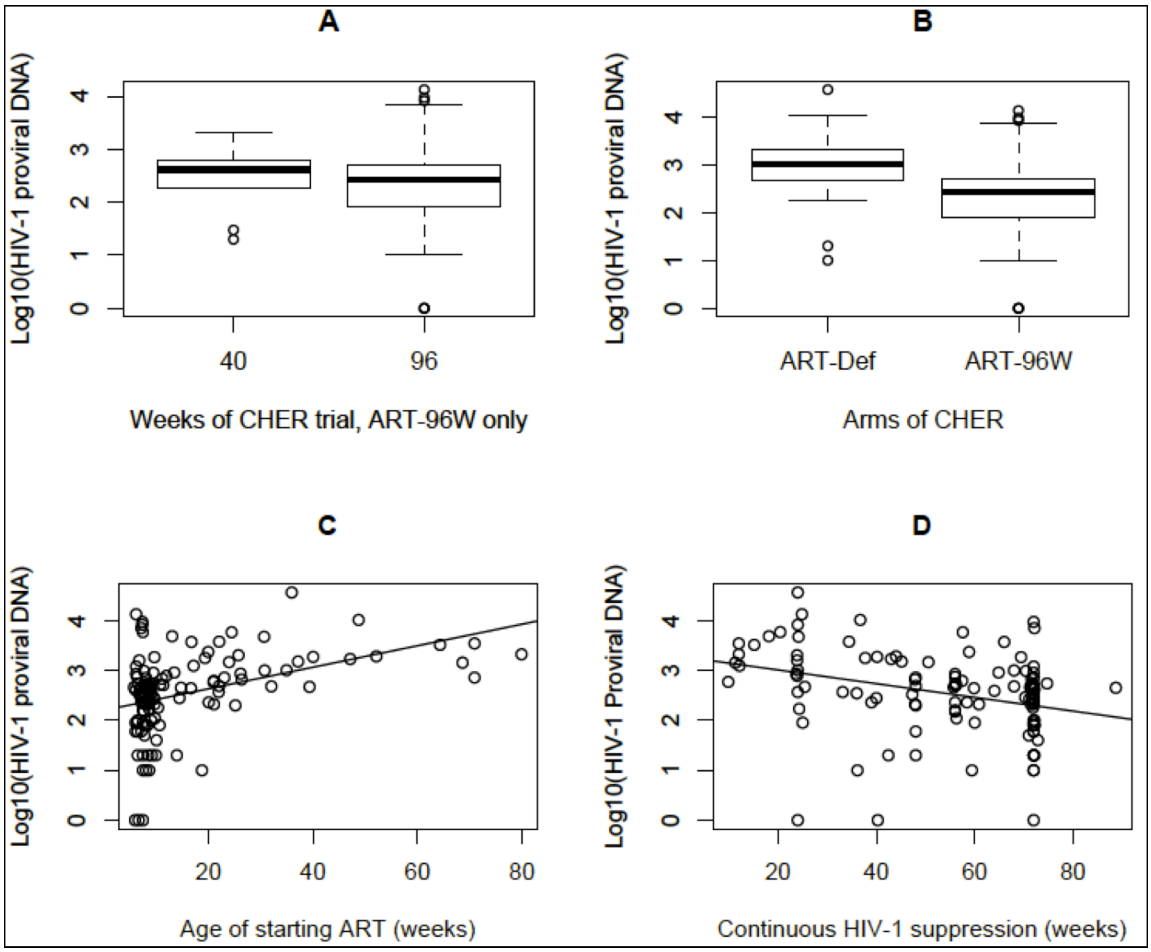
In this chapter, specimens have been used from CHER to describe the differences in the peripheral HIV proviral DNA between early and deferred ART, and where possible compare continuous and interrupted ART. The effect of ART-interruption, the pattern of decline of HIV proviral DNA in patients on continuous ART and any predictive factors that may influence the magnitude of decline have been explored such as serostatus, quantitative HIV-antibody, baseline CMV DNA, and neurocognitive outcome.

8.2 Results

8.2.1 HIV proviral DNA in early versus deferred ART

Between weeks 40 and 96, proviral DNA in ART-96W decreased from median 420 [IQR 190-640] to 270 [IQR 80-510] HIV proviral DNA copies per 10^6 PBMCs (Figure 8.1A). By week 96 3/73 (4%) children had no detectable HIV proviral DNA. Estimates of the peripheral HIV-reservoir from quantification of HIV proviral DNA were compared between ART-Def and ART-96W at 96 weeks on trial. Weeks spent on ART by trial week 96 was significantly less in ART-Def (median 81 [IQR 70.3–85.9]) versus ART-96W (96 [IQR 96-96] weeks, $p<0.0001$), and there was significantly more HIV proviral DNA in ART-Def (median 1000 [IQR 480–2020]) compared to ART-96W (270 [80–510] copies of provirus per 10^6 PBMCs, $p<0.0001$, Figure 8.1B). HIV proviral DNA is higher in those starting ART at an older age ($p<0.0001$, Figure 8.1C) and lower in those with a longer duration of continuous HIV RNA suppression below 400 copies/mm^3 ($p<0.0001$, Figure 8.1D).

Figure 8.1: HIV proviral DNA at week 96 of the CHER trial.



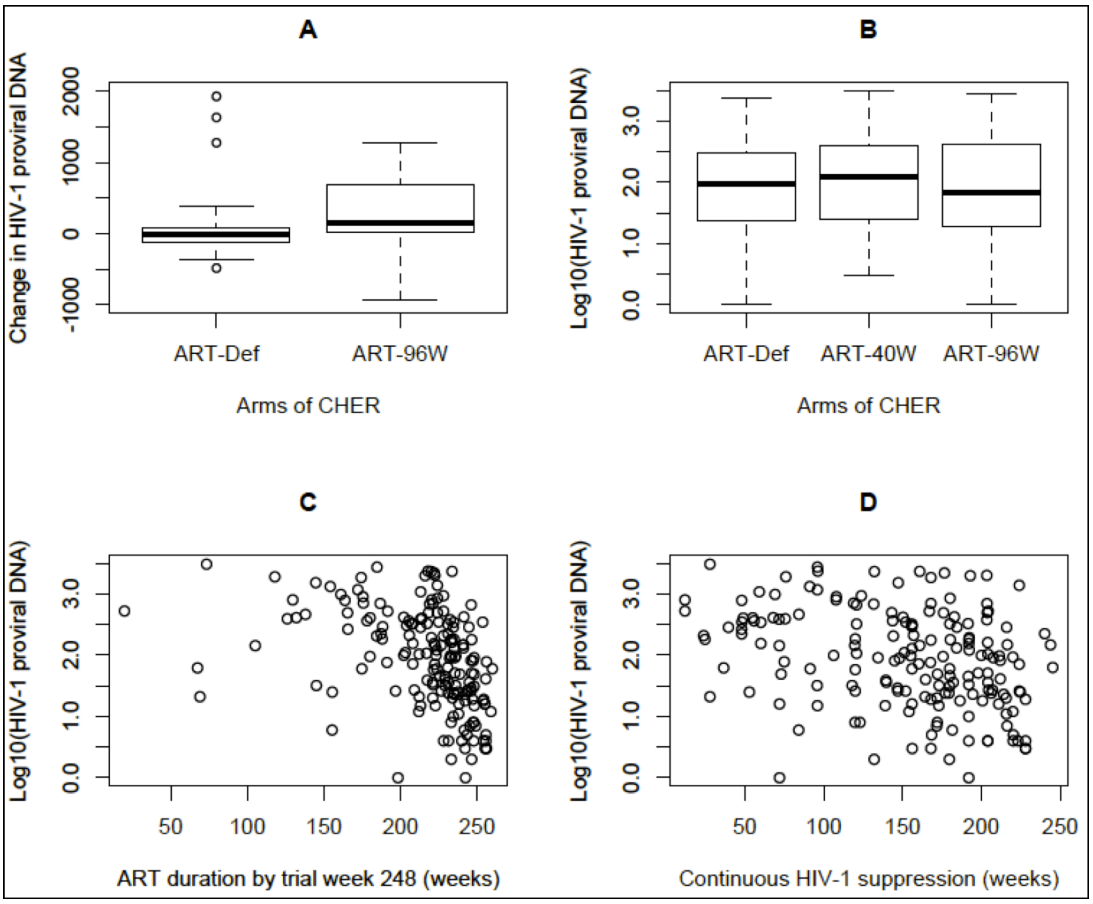
Plot A: HIV proviral DNA from ART-96W at trial week 40 (n=9) and 96 (n=73), no difference in levels of proviral DNA (p=0.7). Plot B: HIV proviral DNA in ART-Def (n=45, median 1000 [IQR 420-2020]) versus ART-96W (n=73, median 270 [IQR 80-510]) at trial week 96 (p<0.0001). Plot C: HIV proviral DNA at trial week 96 by age of starting ART (n=118, correlation coefficient = 0.4, p<0.0001). Plot D: HIV proviral DNA at trial week 96 by weeks of continuous HIV RNA suppression below 400 copies per mm^3 (n=118, correlation coefficient = -0.4, p<0.0001). Y-axes represent HIV proviral DNA (Log10 copies per 10^6 PBMCs).

8.2.2 ART-interruption and HIV proviral DNA

Time off ART during interruption was unrelated to levels of HIV proviral DNA at 96 weeks in ART-96W (p=0.7). We examined change in proviral DNA between 96 and 248 weeks of the trial. In 17 paired samples from ART-96W who underwent ART-interruption for a median 45.1 weeks [IQR 34.4–80.4] there was an overall trend towards increasing proviral DNA (median +250 [IQR +1530 to +6860] copies of provirus per 10^6 PBMCs, t-test p=0.07, Figure 8.2A). In contrast, in 41 paired samples from ART-Def on continuous ART, there was no significant change in proviral DNA (median decrease 140 [IQR -1190 to +900] copies of provirus per 10^6 PBMCs, t-test p=0.6).

The effect of time off ART in the first (ART-Def, n=75), second (ART-40W, n=56) or third (ART-96W, n=44) year of life was assessed by comparing HIV proviral DNA between the three different treatment strategies at 248 weeks. In the children who fulfilled the selection criteria at 248 weeks (≥24 weeks of ART and two consecutive undetectable plasma HIV RNA ≥3 months apart), there was no significant difference between the three arms in total time spent on ART from enrolment until 248 weeks (median 229 [IQR 215-235] weeks for ART-Def, 226 [203-244] for ART-40W, 224 [183-248] for ART-96W; ANOVA p=0.5). Similarly, there was no significant difference at 248 weeks between the 3 arms in HIV proviral DNA (median 950 [IQR 240-2950] copies of provirus per 10^6 PBMCs for ART-Def, 1250 [260-3870] for ART-40W, 690 [200-4080] for ART-96W; ANOVA p=0.5, Figure 8.2B). In univariate analyses, HIV proviral DNA decreased with time on ART (p<0.0001, Figure 8.2C) and weeks of continuous HIV RNA suppression below 400 copies/mm^3 (p<0.0001, Figure 8.2D). Greater duration of ART received by 248 weeks was significantly associated with lower levels of proviral DNA, however it is clear from the plots that some children had low levels of provirus despite a relatively short duration of ART or weeks of continuous HIV RNA suppression and others had high proviral DNA, despite longer time on ART.

Figure 8.2: HIV proviral DNA at week 248 of the CHER trial.

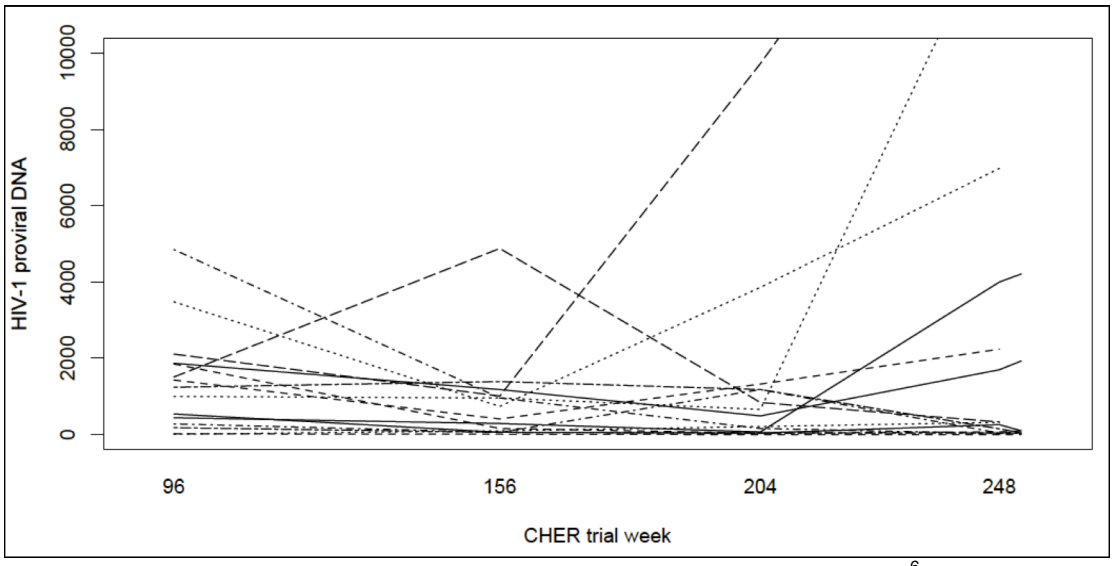


Plot A: Change in HIV proviral DNA from trial week 96 to 248 by CHER trial arm, comparing continuous ART (ART-Def, n=41 children who had samples stored from both weeks 96 and 248) with interrupted ART (ART-96W, n=17 children with samples from both weeks 96 and 248, median interruption 45.1 [IQR 34.4 – 80.4] weeks). Welch's t-test for difference in provirus from 96 to 248 weeks between ART-Def and ART-96W p=0.07. Plot B: HIV proviral DNA in ART-Def (n=75), ART-40W (n=56) and ART-96W (n=44) at 96 weeks of trial (ANOVA p=0.5). Plot C: HIV proviral DNA at trial week 248 by duration of ART received by 248 weeks (n=175, correlation coefficient = -0.35, p<0.0001). Plot D: HIV proviral DNA at trial week 248 by weeks of continuous HIV RNA suppression below 400 copies per mm^3 (n=175, correlation coefficient = -0.24, p<0.0001). Plot A y-axis represents change in quantity of HIV proviral DNA represented in copies of provirus per 10^6 PBMCs and has been shortened to better visualize the data distribution from -17000 to -10000 removing one data-point from ART-Def, and from 28000 to 20000 removing 2 data-points from ART-96W. Plots B-D y-axes represent quantity of HIV proviral DNA (Log10 copies per 10^6 PBMCs).

8.2.3 Determinants of HIV proviral DNA

Fifteen children from ART-Def on continuous ART each had 3-5 measures of HIV proviral DNA available between 84 and 252 trial weeks (Figure 8.3). As a group, HIV proviral DNA declined over the 156 weeks examined. However, one child had a peak at 156 weeks and six children had increasing provirus towards 252 weeks. These six children were all HIV-seropositive at 84 weeks and one child had evidence of a "viral break-through" to 4.8log, which subsequently suppressed. The child with the highest proviral peak had high levels of CD8 T-cell activation (CD8+HLADR+ 85-67%) but much lower CD4+ T cell activation (CD4+HLADR+ 13-10%). There was also high proliferation in both CD4 and CD8 memory T-cells (40% and 50% respectively) and low naïve CD4 T-cells (8-12% of all lymphocytes). In contrast, the child with the lowest reservoir demonstrated comparatively lower levels of activation (CD8+HLADR+ 36%, CD4+HLADR+ 5%), low proliferation in memory T-cells (CD4+Ki67+ 11%, CD8+Ki67+ 17%), and high naïve CD4 T-cells (42%).

Figure 8.3: Pattern of HIV proviral DNA change in 15 children from ART-Def on continuous ART.

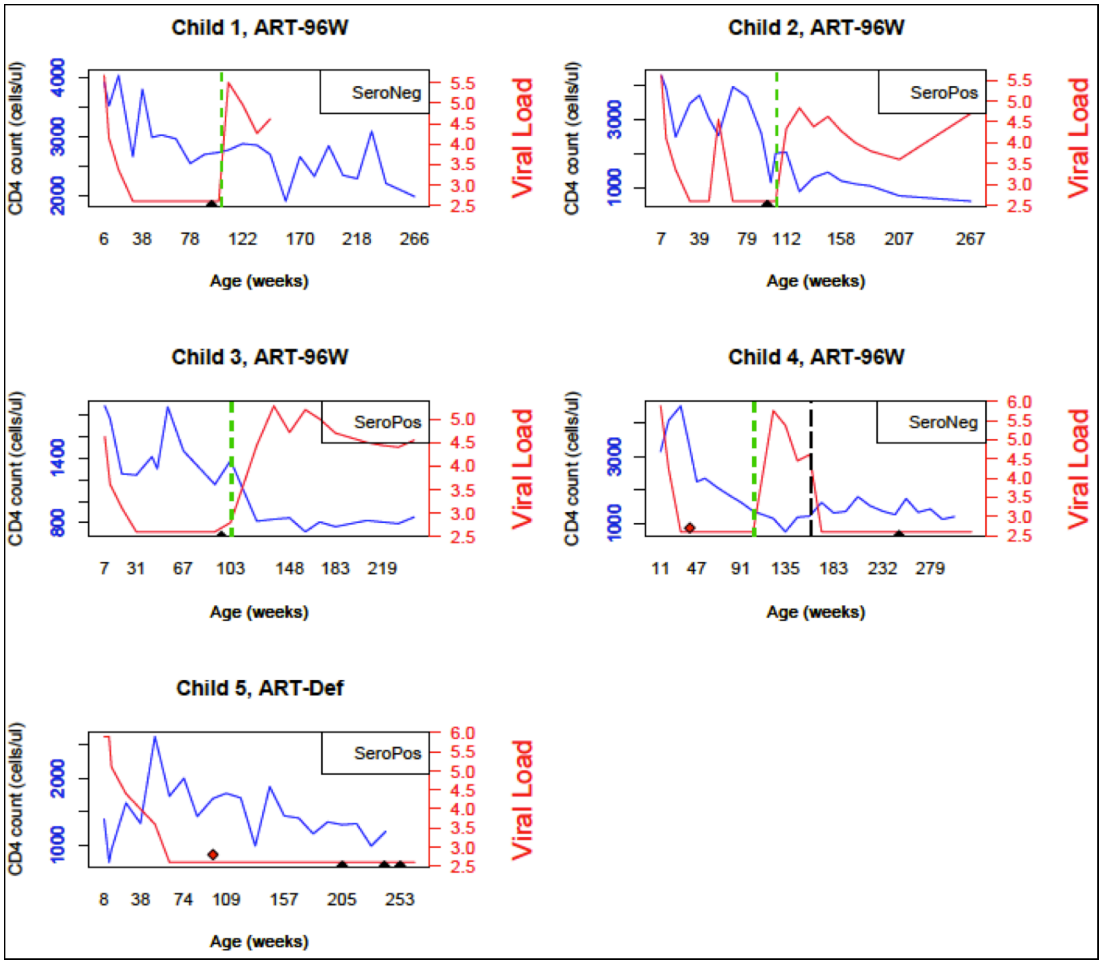


The y-axis represents quantity of HIV proviral DNA copies of provirus per 10^6 PBMCs and has been shortened to better visualize the data distribution from $1.9x10^4$ to $1.0x10^4$.

Of all 334 HIV proviral DNA measurements, 60 values in 47 children were ≤100 copies per 10^6 PBMCs, with 31 of these children having 36 measures ≤50 copies per 10^6 PBMCs including five children with undetectable proviral DNA. These undetectable measures occurred at 96 or 248 weeks of the trial, following at least 96 weeks of continuous ART (further detail shown in Figure 8.4 and Table 8.1). At 96 weeks 23/73 (31.5%) children from ART-96W had HIV proviral DNA <100 copies per 10^6 PBMCs and 3 (4%) had undetectable provirus. The only predictor of HIV proviral

DNA <100 copies per 10^6 PBMCs at 96 weeks in children who received early ART for 96 weeks (ART-96W) was high enrolment CD4 count (p=0.03). However, predictors of undetectable provirus at 96 weeks were: longer duration of HIV RNA suppression (p<0.0001) and lower levels of anti-gp120 IgG (p=0.02). Negative serostatus for HIV-antibody was not predictive (p=0.7).

Figure 8.4: Profiles of the 5 children with an undetectable HIV proviral DNA measurement at any time during the CHER trial.



Blue = CD4 count (cells/μl), Red = Log10 HIV RNA viral load, green-dashed line = ART-interruption at 96 weeks; black-dashed line = restart ART after ART-interruption, black diamond = undetectable HIV proviral DNA, red diamond = <50 copies HIV proviral DNA. Children 1-4 started ART aged 6-7 weeks and interrupted ART after 96 weeks. Child 5 started at 14 weeks of age and did not interrupt ART.

Table 8.1: Characteristics of children with undetectable HIV proviral DNA (copies per 10^6 PBMCs). Naïve CD4 T-cells (CD3+CD4+CD45RA+ of all CD4 T-cells) and proliferation of memory CD4 T-cells (CD3+CD4+CD45RA-Ki67+ of all memory CD4 T-cells) were taken from various time-points from enrolment to trial end.

	Child 1	**Child 2**	**Child 3**	**Child 4**	**Child 5**
CHER arm	ART-96W	ART-96W	ART-96W	ART-96W	ART-Def
HIV proviral DNA* and trial week (w)	<10 at 96w	<10 at 96w	<10 at 96w	<10 at 248w	17 at 96w <10 at 204w <10 at 248w <10 at 252w
Enrolment					
Age (weeks)	5.9	6.6	7.4	7.0	8.3
Viral load (Log cp/ml)	5.7	5.6	4.6	>5.9	>5.9
HIV DNA PCR	Positive	Positive	Positive	Positive	Positive
CMW DNA PCR	Negative	Negative	Negative	Negative	Negative
During the trial					
Age starting ART (weeks)	5.9	6.6	7.4	7.0	13.9
Serology at trial week 84	Negative	Positive	Positive	Negative	Positive
HIV Viral load (cp/ml)	<400 until 96w	<400 with a viral blip to 4.6log at 48w	<400 until 96w	<400 until 248w	<400 until 252w
Range of CD4 counts (cells/ul)	1903-4028	593-4272	716-1888	773-4497	742-2621
Naïve CD4 T-cells (median %)	78.6 (5 values)	74 (6 values)	52.2 (2 values)	91.2 (6 values)	80 (1 value)
Memory CD4 proliferation (median %)	17.2 (5 values)	13.2 (6 values)	13.5 (2 values)	22.2 (6 values)	11.2 (1 value)
ART interruption					
Duration (weeks)	Remained off ART until end of trial (minimum 152 weeks)			56.7	N/A
Plasma HIV viral rebound	Steep rebound to 5.5log then stabilized to 3.3log	Lower initial rebound to 4.3log then remained 4log	Lower initial rebound to 4.4log then fluctuation 4.4 – 5.3log	Steep rebound to 5.8log then dropped to 4.6log	N/A

HIV serostatus and HIV proviral DNA

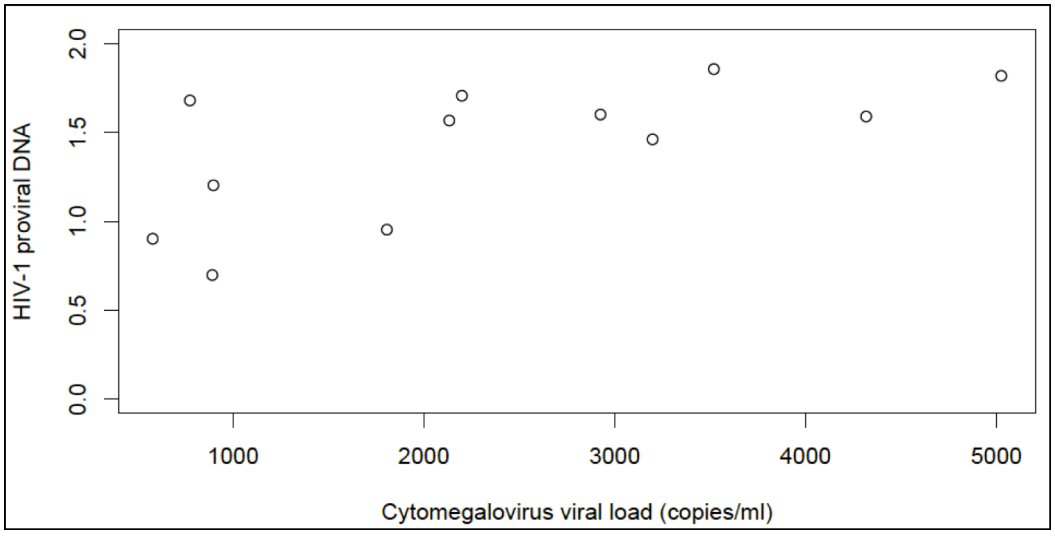
A trend between seronegative status at 84 weeks and lower levels of proviral DNA at 96 weeks was found in ART-Def (n=39, T-test p=0.08) although not in ART-96W (n=68, p=0.92). Quantitative anti-gp120 IgG did not correlate with levels of proviral DNA (correlation coefficient=0.04, p=0.66) in either group. In ART-96W, 33/68 (48.5%) were seronegative at 84 weeks, of which 1/33 (3%) had undetectable provirus and 12/33 (36.4%) had <10 copies of provirus per 10^6 PBMCs at 96 weeks. Two of the three children who had undetectable provirus at 96 weeks were

seropositive; therefore it was not possible to propose a minimum proviral load that might be suggestive of seronegative status. However, the predictive capacity of seronegative status at 84 weeks in estimating proviral DNA in children started on ART at <12 weeks of age (ART-96W, n=68) was as follows: sensitivity of 90.9% and specificity of 8.6% for <100 copies of provirus per 10^6 PBMCs, with decreasing sensitivity and increasing specificity to 36.4% and 77.1% respectively for <10 copies.

Cytomegalovirus and HIV proviral DNA

A positive CMV DNA-status at CHER enrolment did not correspond to high or low levels of HIV proviral DNA reservoir at 96 weeks (median 450 [IQR 100-1000] in CMV-negative versus 380 [IQR 200-610] copies per 10^6 PBMCs in CMV-positive, p=0.6), in ART-Def and ART-96W separately and combined (n=97). However, although numbers were small, high quantitative CMV viral load (>150 copies/ml) was significantly associated with high levels of HIV proviral DNA at 96 weeks (n=12, correlation coefficient = 0.67, p=0.02, R^2=0.45). All children with undetectable HIV proviral DNA at any time during the CHER trial were noted to have undetectable CMV DNA PCR at enrolment (Table 8.1).

Figure 8.5: Cytomegalovirus viral load and HIV proviral DNA at 96 weeks of CHER.



High levels of CMV at enrolment of CHER were significantly associated with higher HIV proviral DNA (n=12, correlation coefficient = 0.67, p=0.02, R^2=0.45). Y-axis represents quantity of HIV proviral DNA (Log10 copies per 10^6 PBMCs).

8.2.4 Neurocognitive state and HIV proviral DNA

HIV proviral DNA quantification from 96 and 248 weeks of the trial (n=29 and 65) were examined to explore whether peripheral measures of HIV proviral DNA might reflect HIV-associated CNS disease and impact upon neurocognitive performance (Tables 8.2 and 8.3).

Table 8.2: Characteristics of the CHER participants that had a Griffith's assessment and HIV proviral DNA quantification at trial week 248 (n=65). *Statistical significance in age of ART-initiation as per CHER trial design.

	ART-Def	**ART-40W**	**ART-96W**	
No.	23	25	17	
Male	39%	44%	53%	
	Median [IQR] (range)			**p-value**
CD4%	38.2 [36 – 44.2]	36.8 [31.5–39.5]	34.2 [31.3–41.5]	0.07
CD4 count	1176 [947-1473]	1157 [1036-1308]	1087 [894-1426]	0.76
Continuous viral suppression (weeks)	168 [120 - 204]	151 [84 - 173]	180 [140 - 212]	0.20
ART start age (weeks)	33.7 [20.4-40.7]	7.7 [7.3 – 9.3]	7.7 [7 – 9.1]	<0.0001*
Time off ART (weeks)	33.7 [20.4–40.7]	36.9 [18.9–59.3]	21.7 [7.6–43.6]	0.10
Total ART (weeks)	223 [216 - 234]	226 [207 - 245]	244 [221 - 255]	0.14
Proviral DNA (copies/10^5 cells)	36 [21 – 103] (4 – 2,346)	49 [18 - 101] (3 – 385)	26 [16 - 46] (0 – 402)	0.11 (3 arms) 0.16 (Def vs 40W) 0.09 (Def vs 96W) 0.16 (40W vs 96W)
	Griffith's scores – Median [IQR]			**p-value**
Age at Griffith's (weeks)	264 [261 – 268]	260 [260 - 264]	262 [258 - 267]	0.06
Locomotor (%)	73 [67 – 81.5]	70 [64 - 76]	72 [66 - 78]	0.37
Personal and social (%)	68 [66 – 74]	66 [62 - 70]	66 [62 - 66]	0.19
Language (%)	56 [51 – 64]	54 [48 - 58]	56 [50 - 60]	0.61
Eye-hand coordination (%)	56 [50 – 57]	52 [44 - 56]	52 [48 - 58]	0.11
Performance (%)	58 [51 – 58]	50 [46 - 56]	50 [48 - 54]	0.46
General Griffiths (%)	60 [57 - 64]	57 [53 - 60]	57 [54 - 61]	0.10
Visual Motor integration (VMI)	91 [84 - 95]	89 [85 - 93]	89 [81 - 91]	0.62
Visual standard	76 [63 - 89]	78 [74 - 89]	74 [63 - 84]	0.59
Motor standard	96 [86 - 100]	92 [87 - 96]	97 [92 - 100]	0.81

The only significant finding was that levels of HIV proviral DNA at 96 weeks were correlated with motor standard score (correlation coefficient = -0.4, p-value=0.03, R^2=0.12), i.e. higher levels of HIV proviral DNA at 96 weeks were associated with a poor motor standard (fine-motor coordination). However by 248 weeks this association was no longer present.

Table 8.3: Correlation of Griffith's assessment variables with levels of HIV proviral DNA at 96 and 248 weeks of the CHER trial. Pearson's correlation coefficient (r). *Indicates statistical significance at <0.05.

Neurocognitive variable	HIV proviral DNA at 96 weeks (n=29)		HIV proviral DNA at 248 weeks (n=65)	
	r	p-value	r	p-value
Locomotor quotient	-0.22	0.25	-0.09	0.49
Language quotient	-0.09	0.65	0.18	0.16
Personal and social	0.29	0.12	0.03	0.78
Eye-hand coordination	-0.05	0.77	-0.0005	0.99
Performance	-0.008	0.97	-0.12	0.34
General Griffiths	-0.12	0.54	-0.04	0.74
VMI standard	-0.20	0.31	-0.04	0.76
Visual standard	-0.21	0.29	-0.09	0.47
Motor standard	-0.4	0.03*	-0.08	0.54

8.3 Discussion

HIV proviral DNA was lower at 96 trial weeks in children receiving early ART for 96 weeks compared to deferred ART until clinically or immunologically indicated. Earlier age of ART-initiation and longer duration of continuous HIV RNA suppression were significantly associated with lower levels of HIV proviral DNA, thus supporting the results from smaller studies [202, 218, 219]. However, there was no difference in HIV proviral DNA levels between the three ART-strategies of the CHER trial at 248 weeks. This suggests that the benefit gained from early ART is lost by interrupting ART, or alternatively, the disadvantage of starting ART later might be offset by continuous ART.

There is an inherent selection bias in this study since more children died in ART-Def, meaning that children who died contributed fewer reservoir results. Clinically unwell children may have been less likely to have an aliquot of PBMCs or plasma stored. However, these biases might underestimate the associations seen above as unwell children may be more likely to have uncontrolled HIV viraemia and presumably more integrated HIV proviral DNA. The effect of duration of HIV infection and age of ART initiation on HIV proviral DNA levels cannot be distinguished, since these two variables are intrinsically related.

While early ART and sustained viral suppression are clearly important determinants of the viral reservoir, we observed different patterns of virological dynamics within individuals. As both Figures 8.1 and 8.2 illustrate, a few children have low proviral reservoirs despite relatively short durations of HIV RNA suppression, and some have high reservoirs despite long-term ART. When examining the trajectories of HIV proviral DNA in the children on continuous ART, it is clear that some have a variable course despite apparent sustained virological suppression. This has been described previously [214] and could be explained by interval viral load testing not capturing “viral blips”. It may also be due to homeostatic proliferation in the absence of viral reactivation [246], or by low-level antigen-driven stimulation, T-cell activation and subsequent proliferation of HIV-infected memory CD4+ T-cells that harbor latent integrated HIV [213].

High CMV viral loads at CHER enrolment were associated with high levels of HIV proviral DNA at 96 weeks, as seen in a adult study [233], while all children with undetectable HIV proviral DNA were CMV DNA negative at enrolment. High levels of T-cell activation and proliferation were also observed in individuals with high HIV

proviral DNA. This raises the possibility that immune activation, potentially due to co-infections such as CMV, may contribute to the formation and accumulation of the latent reservoir. High naïve CD4 T-cells were observed in individuals with low levels of HIV proviral DNA perhaps reflecting good thymic output. Although little can be concluded from these observations, there appears to be a complex interplay between virological and immunological dynamics and levels of HIV proviral DNA, likely to be influenced by ART adherence, co-infections, timing of HIV-transmission and initial maternal viral burden.

High levels of HIV proviral DNA at 96 weeks were shown to be associated with significantly lower standards or fine-motor coordination using the Griffith's assessment. This association disappeared by 248 weeks suggesting that the deficit could resolve with time, or that it might not be a true finding. Further analysis to explore this association is needed since multiple statistical comparisons were done in this analysis and the association might have therefore occurred due to chance rather than biological plausibility.

Predictors of low or undetectable provirus included longer duration of HIV RNA suppression, high CD4 count at enrolment, lower quantity of HIV-specific antibody but not negative serostatus. The lack of absolute correlation between serostatus or antibody quantity and proviral DNA levels imply that the dynamics of reservoir decline may not be solely reliant on adequate viral suppression, as observed previously[205], although it could suggest that interval viral load testing might not be a reliable way of indicating continuous viral control.

Five individuals had one or more measures of undetectable HIV proviral DNA, of whom three underwent ART-interruption and remained off ART by end of trial. One child interrupted ART for 56 weeks and the fifth child did not interrupt as she was randomised to ART-Def. All children who interrupted ART exhibited HIV RNA rebound during interruption, inferring that undetectable provirus, at least at the level of detection for this study, cannot indicate cure. The measurement of HIV proviral DNA alone, or with HIV-antibody status, is not sufficient to predict the outcome of children who stop ART. Improvements in the sensitivity of viral DNA detection, combined with assays that ascertain levels of replicative virus may be more informative, however such assays require large volumes of blood, which is a barrier in young children. Further work is required to understand the complex relationship between HIV viral dynamics and immune function to help identify children who may be functionally cured or amenable to novel treatment modalities.

In summary, this study demonstrates reduced levels of HIV proviral DNA in children started on early ART. Levels of HIV proviral DNA are strongly associated with earlier ART-initiation and longer continuous virological suppression, however HIV proviral DNA levels prior to ART-interruption do not predict time tolerated off ART. ART-interruption appears to replenish the reservoir to that of a child starting ART later i.e. >12 weeks of age. Pre-ART events such as reduced naïve CD4 T-cells, co-infections and immune activation may be import determinants of HIV proviral DNA levels and provide potential targets for adjunctive reservoir reduction strategies. These findings provide valuable insights into the development and dynamics of the HIV reservoir and support early ART with sustained virological suppression to limit levels of HIV proviral DNA in children.

Chapter 9

Conclusions

The CHER trial demonstrated a hugely significant benefit of early ART on morbidity and mortality of paediatric HIV and has had a fundamental impact upon treatment guidelines and survival worldwide. While the clinical outcome is indisputable, the immunological and virological mechanisms by which this survival advantage occurs are not well understood and have been the focus of this thesis. More detailed knowledge of these dynamics has the potential to contribute towards improving strategies to further reduce morbidity and mortality, establish more convenient and cost-effective care, optimise immunological health and strengthen approaches towards functional cure in certain individuals.

The prerequisite to enhanced understanding of these immunological and virological mechanisms is a detailed knowledge of the paediatric immunology of a comparative HIV-uninfected population. The Child Wellness Clinic was created for this purpose and has established a set of local haematological and immunological reference intervals for the South African paediatric population. The comparison of these immunological data with 3 similar studies from the US and Europe reinforced the need for population-specific reference intervals. However, the main finding of these comparisons was the increased naïve to memory T-cell ratio in early childhood, presumably due to increased environmental pathogen exposure as reflected in the elevated activation markers and levels of natural killer cells compared to their US and

European counterparts. While providing insight into the developing paediatric immune system within an African context, the long-term health implications of these findings certainly require further study.

The immunological and virological laboratory analysis performed during this fellowship used cryopreserved PBMCs and plasma from the CHER trial and the CWC cohort. This presented the opportunity to explore the effect of cryopreservation on immunological markers by comparing a large number of specimens that were processed by both fresh whole blood assay and using cryopreserved PBMCs. The preliminary work comparing fresh and cryopreserved PBMCs revealed no significant differences in a range of cell-surface markers, however numerous statistically significant differences were found when comparing a fresh whole blood assay with cryopreserved PBMCs. Although not all statistically significant differences were likely to be clinically relevant, this analysis suggested methodological differences play a prominent role and caution should be applied when comparing studies with differing technical procedures, and in studies using cryopreserved specimens unless appropriate controls are used. In the remaining analysis done in this thesis, immunological results from cryopreserved specimens were only compared to other cryopreserved specimens from relevant control groups.

The measures of thymic output obtained from the cryopreserved specimens of the CWC cohort were used to formulate a non-linear regression curve to delineate the change in thymic output during childhood. Although the shape of the curve clearly differed to that anticipated by the mathematical model, there are numerous reasons why this might be including a potential feedback mechanism to the thymus from increased naïve to memory T-cell conversion as demonstrated by the CWC immunological analysis.

The CD4 and age-adjusted thymic output analysis from both the healthy children from the CWC and the combination of CHER arms illustrated that higher absolute CD4 counts were significantly associated increased thymic output, highlighting the importance of preserving thymic output in children to optimise their potential for CD4 reconstitution and long-term immunological health. These findings were reinforced and further explored by the analysis of the impact on thymic output of the different ART-strategies used by the CHER trial.

Immunological changes in HIV-infected children with or without ART

Overall the ART-strategies of early ART for 96 weeks (ART-96W), followed by ART-interruption until clinical/immunological deterioration, appeared to preserve thymic output in the early childhood years to that of a healthy HIV-uninfected child. The ART-strategy of ART-96W appeared to be more beneficial long-term over deferred ART followed by continuous ART (ART-Def), and early ART continued for 40 weeks with subsequent ART-interruption (ART-40W). Examining the individual thymic output trajectories and combined regression curve of ART-96W shows thymic output to be high in the first few months of life. This may be a consequence of the initial effects of untreated HIV-infection, bringing the thymic output peak earlier than expected in the first year of life, hypothetically due to a feedback mechanism from CD4 T-cell death plus antigen-stimulated naïve to memory T-cell conversion. High levels of thymic output may be unsustainable and result in depleted thymic output even though ART is initiated around that time. Thereafter, the benefits of ART in suppressing HIV RNA and reducing associated CD4 T-cell death are seen with a corresponding increase in thymic output. This pattern is replicated by the trajectories and analysis exploring ART-interruption and the effect of deferred ART, alongside observations of low thymic output at the trial-defined endpoints, and in cases of pneumonia. The association between low thymic output and poor clinical outcome, such as acquiring pneumonia, may be due to limited functionality of circulating naïve T-cells from a restricted TCR repertoire however this inference requires verification.

Despite on-going ART, the immunophenotype comparisons between HIV-uninfected and HIV-infected children suggest immune activation persists and more needs to be done to restore immunological health to that of an HIV-uninfected child. It may be that HIV RNA viral load is inadequately suppressed, and viral load testing strategies, if available, are not sensitive enough or do not capture transient increases in plasma viral load. This requires more detailed scrutiny, however it has not been possible in this thesis since the viral load data from CHER was incomplete at time of analysis. Some caution should be applied to these findings since the analyses were done on cryopreserved PBMCs, and although all comparisons done were between cryopreserved specimens, the cryopreservation-thawing process might have influenced the differences detected.

The immunopathogenesis of HIV is largely due to accelerated destruction combined with impaired regeneration of CD4 T-cells. While ART successfully reduces CD4 T-cell destruction and timely ART-initiation may prevent impairment of thymic output, there is a dearth of adjunct strategies to repair thymic damage and optimise

immunity. In addition to the relationship seen between CD4 count and thymic output, it is possible that several other immunological markers such as activation, proliferation or naïve/memory ratio may also be associated with thymic output, and plausibly involved in a feedback mechanism to the thymus, as has been seen with T-regulatory cells [247]. However, further complex analyses are needed to unravel these associations and thereafter ascertain clinical approaches to facilitate the function of organs that generate naïve T-cells, such as the bone marrow, thymus, and mucosal immune system.

The naïve B-cell and thymic output analyses revealed similar peaks in output within the first year of life in both sets of children from the CWC and ART-96W. The magnitude of the naïve B-cell peak was far higher and sustained for longer in ART-96W compared to the healthy HIV-uninfected controls, yet during this time thymic output appeared to be falling as a consequence of untreated HIV. These results could infer the intrinsic effect of uncontrolled HIV on the bone marrow; or alternatively, might suggest that a feedback mechanism could exist during times of immunological pressure on the thymus to thus stimulate the bone marrow to increase early lymphocyte progenitors and maintain thymic output. While intriguing results, further research is needed to validate these results and explore the mechanisms involved.

Quantity of HIV-specific antibody was not found to be directly related to naïve B-cell output but instead highly correlated with cumulative viral load exposure and therefore more likely to be due to HIV antigen-stimulation than any B-cell deficit or dysfunction. Although all children tested in the antibody substudy had detectable anti-gp120 IgG, almost half children starting ART within 12 weeks of birth and 6% of those starting at 12-24 weeks were seronegative by automated serology or rapid antibody tests at approximately 2 years of age. In view of these findings, commercial antibody tests should not be used to confirm HIV status among children already diagnosed and started on ART at less than 6 months of age. This has clinical implications since the confirmation of diagnoses are required for several reasons particularly for children started on ART with presumptive diagnoses in resource-limited settings and false negative serology may result in inappropriate cessation of ART.

A further implication of the seronegative status is the growing attention on approaches towards achieving functional cure in HIV-infected children. HIV proviral DNA is the current gold standard for diagnosis, however reduced levels of HIV proviral DNA were demonstrated in children started on early ART in this study. Lower

levels of proviral DNA were found to be strongly associated with earlier ART-initiation and longer continuous virological suppression. However, HIV proviral DNA quantity did not predict time tolerated off ART and ART-interruption appeared to replenish the reservoir. Pre-ART events such as reduced naïve CD4 T-cells, co-infections and immune activation may be import determinants of levels of proviral DNA and provide potential targets for adjunctive therapy in reservoir reduction strategies. These findings provide valuable insights into the development and dynamics of HIV proviral DNA and lend support to the early use of ART with sustained virological suppression to limit the viral reservoir in children.

Limitations and future studies

Alongside an enhanced knowledge of the immunological dynamics of healthy children in a disease-burdened setting, these novel insights develop the understanding of the influence of HIV and ART on thymic and naïve B-cell output, antibody status, proviral DNA reservoirs and a range of immunological parameters. A vast amount of data has been generated during this fellowship, however the analysis so far has been largely descriptive. The CHER trial was a randomized controlled trial, however there were numerous potential biases such as missing samples since more children died in ART-Def, multiple confounding factors involved in ART interruption analysis, incomplete viral load data, and that only virally suppressed children could be used for proviral DNA analysis. While the current analysis of the antibody and proviral DNA studies may be directly applicable to clinical guidelines, there is great potential for more complex combined analyses using sophisticated statistical techniques and mathematical modeling that are beyond the scope of this thesis but that I intend to pursue with the assistance of colleagues in the future.

Further long-term studies are needed to establish the relationship between thymic and naïve B-cell output with clinical health. Decline in naïve T-cell output has been associated with reduced T-cell diversity and thereby increased susceptibility to infection, autoimmune disease and cancer [5]. Longitudinal analysis of individuals would more accurately characterise the trajectories of thymic and naïve B-cell output. A valuable longitudinal study would be one that quantifies thymic and naïve B-cell output with T-cell and B-cell receptor diversity alongside the clinical and immunological condition of the child as they progress throughout childhood, aiming to determine what is the range of optimal thymic and naïve B-cell output required for good clinical health. It would be necessary to identify crucial factors associated with establishing and maintaining thymic and naïve B-cell output such as nutrition, genetics, and exposure to a range of environmental pathogens.

Laboratory studies in animals might further explore the feedback mechanism postulated whereby peripheral destruction of CD4 T-cells or naïve-to-memory T-cell conversion hypothetically results in increased output of naïve CD4 T-cells from the thymus, and potentially drives increased output of early lymphocyte progenitors from the bone marrow. Proof that this mechanism exists, and whether it changes with age, may be clinically important and might even inform strategies on ART-interruption i.e. if higher thymic output in early childhood years was related to long-term improved clinical health, therapeutic mechanisms to increase thymic output might be indicated such as the use of IL7; alternatively if the mechanism is age-dependent and diminishes with age it is plausible that limited ART-interruption might only be feasible in earlier years when the mechanism exists.

A follow-up study of the CHER trial is underway and over the next five years I will continue to measure the participants' thymic output, immunological profile, serostatus and HIV-specific antibody. This will give a greater longitudinal perspective on the effect on thymic output of early and deferred ART, and the impact of ART interruptions in the second or third year of life. The immunological data generated with be analysed with data from clinical events, neurocognitive function, and detailed virology including infectious virus recovery assays, proviral DNA and viral loads.

Summary

The initial aim of my fellowship was to describe the influence of HIV and ART on thymic output in infants and children. As the fellowship progressed, opportunities arose to utilise the infrastructure of my study to explore other relevant aspects of the immunology and virology of HIV-infected children. I established a healthy child clinic to acquire blood specimens to use as HIV-uninfected controls, and alongside the specimens available from the randomised controlled CHER trial I had access to a large invaluable biological resource. I have learnt and built upon existing laboratory methods, and have successfully created a rich source of immunological data, including normal heamatological and immunological reference intervals for South African children. I have then collaborated with colleagues with skills in mathematical modeling to explore and analyse these data. Therefore the studies of this thesis include immunological profiles of healthy HIV-uninfected and HIV-infected children, the effect of cryopreservation on immunological markers, the effect of early ART on serostatus, the impact of CHER ART-strategies on levels of HIV proviral DNA, and the influence of HIV and ART on thymic output and also naïve B-cell output. These studies are largely descriptive and infer much potential for further investigation, however it is hoped that future work might build upon these findings to further the

knowledge of the child's developing immune system and its response to infection or immunodeficiency, and that this understanding might contribute to advancing ART-strategies to optimise the immunological health of HIV-infected children.

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Appendices

APPENDIX I: Child Wellness Clinic poster and flyers

Child Wellness Clinic

Wesbank Health Centre

You and your baby are invited to attend the "Infant Wellness Clinic" every Tuesday and Wednesday from August 2012 onwards. We would like to ask you if you would agree to take part in a research study to investigate the immune system in well infants.

At the "Infant Wellness Clinic" your child will have the following:

- Clinical review and medical examination
- Height and weight measured
- Have one blood test
- Given nutritional supplements
- Given a "Road to Health" booklet if you do not have one already
- Reimbursement for your time and travel

Your child may take part in the study if he/she is healthy and less than the age of 5 years. If your child is currently unwell or has not had all his/her routine vaccinations, he/she may not be able to take part, however, we will treat him/her if he/she is unwell and administer their missing vaccinations if possible.

The blood taken will be tested for the number of red and white blood cells, HIV-antibody and to measure the production of immune cells. For more detailed information please come along to Wesbank clinic health centre or you can contact me, Dr Helen Payne, on hpayne@sun.ac.za or 0833 633 057.

APPENDIX II: Child Wellness Clinic patient information / consent form

PATIENT INFORMATION LEAFLET AND INFORMED CONSENT FORM

Centre: Children's Infectious Diseases Clinical Research Unit, Tygerberg Children's Hospital
Study number: M12/01/005
Sponsored by: The Wellcome Trust
Principal Investigator: Dr Helen Payne

TITLE OF THE STUDY: THYMIC OUTPUT IN INFANTS AND YOUNG CHILDREN

This information is provided to help you decide whether to take part in this clinical research study. Please read this form carefully and ask the study doctor or the study staff to explain any words or procedures you do not understand.

Dear parent or guardian,

Hello, my name is Helen Payne and I am a children's doctor and research fellow at Tygerberg Hospital. I would like to invite your child to take part in a research project. If your child is healthy then he/she can participate in the trial. I am looking for children who are NOT infected with HIV to take part in this study.

The study will help us, as doctors, understand more about the way in which HIV affects the immune system of babies and young children. A better understanding will mean we will be able to improve the care we give to babies and children with HIV to help them have a healthier life.

The following sheets explain the project in more detail and are for you to keep. When you have read it, or someone has read it to you, I or one of my colleagues will be pleased to answer any questions you have. If you are happy to take part then you will need to sign the consent form when you attend the "Infant Wellness Clinic". No information from the study will reveal your or your child's identity, and you can withdraw from the study at any time.

I thank you in advance for your time and help.
Yours sincerely,

Dr Helen Payne

WHAT IS THE STUDY AND WHY ARE WE DOING IT?

Although your child may not have HIV, HIV infection in children is still a major problem in South Africa. Although treatment with medicines against HIV (HAART) has saved many lives, there are still important questions we do not know the answers to. We know we should start HAART as soon as possible after birth in children who are HIV-infected; however, we do not know exactly how long we should continue HAART and when it is safe to have a treatment break. We hope the results of this study will provide information that will help to answer these questions. This information could be important for HIV management worldwide.

HIV weakens the immune system and makes children vulnerable to serious infections. The immune system is controlled by a gland called the thymus. However it is not understood how the thymus reacts to HIV-infection or treatment. In this study we will investigate how HIV and HAART affect this gland and the production of immune cells (what is called thymic output).

WHY ARE YOU ASKING MY CHILD TO TAKE PART?

In this study we need a group of babies and children who do NOT have HIV to compare with children who do have HIV. It will also help us define what are normal immune cells (cells which fight infection) in this age group because this is something that is not currently known.

WHAT WILL HAPPEN AT THE "CHILD WELLNESS CLINIC"?

At the "Child Wellness Clinic" you will be asked to give us your address and phone number (if possible). Your child will have the following:

- Clinical review and medical examination
- Height and weight measured
- Vaccination review and catch-up if needed
- Have one blood test
- Given nutritional supplements
- Review and update of your "Road to Health" booklet
- You will be reimbursed for your time and travel

CAN MY CHILD DEFINITELY TAKE PART?

Your child may take part in the study if he/she is healthy and is not unwell at the time. If your child is unwell or severely malnourished, she/he will be treated at the health center or referred to hospital if necessary. If your child has not had his/her vaccinations according to the national schedule, we may offer to give his/her vaccines if appropriate or arrange another time to do so. If this is the case, he/she will not be able to take part in the study but may attend the clinic for clinical review, examination, height and weight measurement and supplements.

WHAT ARE THE BLOOD TESTS FOR?

Your child will have one blood test (2-3 mls or 1 teaspoon of blood). This will be used to:

1) Check the level of red and white blood cells (a full blood count)
2) Test for HIV
3) To measure the cells of your baby's immune system and thymic output

HOW WILL I KNOW THE BLOOD TEST RESULTS?

You will need to wait 20-30 minutes until the result is available. You may become worried or anxious while waiting for the results of your child's HIV test. You will have counseling sessions with a trained counselor to help you with any feelings or questions you may have.

WHAT IF MY BABY'S BLOOD TEST IS HIV-POSITIVE?

If the blood taken has tested positive for HIV-antibody, it usually means the baby's mother has HIV. We will perform a second test on the blood (called HIV proviral DNA), which will tell us whether your baby has HIV, or not. If you, or both you and your baby, have HIV we will refer you to the local family HIV clinic where you will be assessed for starting treatment (HAART).

WHAT ABOUT CONFIDENTIALITY?

Information about your HIV status, your child's HIV status, your child's care in the clinic and his/her blood test results will be stored in locked cabinets. All medical information collected during the study will be treated as confidential. Any information that is used for research or publication purposes will be kept confidential and will not have your or your child's name recorded on it. The Health Research Ethics Committee may check that these records are kept correctly.

YOUR CHILD'S PARTICIPATION IS VOLUNTARY

Your child's participation is voluntary. You can refuse to consent to this study and even if you sign the consent form, you can withdraw your consent at any time. Approximately 500 children will take part in this study.

WHAT ARE THE RISKS TO MY CHILD?

There are no serious risks to your child from this study. During the collection of blood samples, your child may experience light-headedness, pain, bleeding and/or bruising at the site on the arm where blood is taken. Blood clots may form and infections may occur, but this is unusual. Fainting may occur during or shortly after having blood drawn.

WHAT DO I DO IF I HAVE QUESTIONS OR PROBLEMS?

Thank you for taking the time to consider this study. If at any time you have any questions during the study, please do not hesitate to contact myself (Helen Payne) or any of my colleagues.

CONTACT DETAILS:

Children's Infectious Diseases Clinical Research Unit
Ward J8, Tygerberg Children's Hospital
University of Stellenbosch
Francie van Zijl Avenue
Parow, 7505, Cape Town, South Africa
hpayne@sun.ac.za
Tel: 0833 633 057

If you would like any information regarding your child's rights as a research participant, or have complaints regarding this research study, you may contact:

The Chairperson
Human Research Ethics Committee
University of Stellenbosch
Tel: 021 938 9075

This independent committee has been established to help protect the rights of research participants and gave written approval for the study protocol.

THYMIC OUTPUT STUDY: INFORMED CONSENT SIGNATURE PAGE

Name of participant: _____

Study registration number: _____Date of Birth:_____

To parents/legal guardians, please TICK the box if the answer to the question is YES:

1) Have you read or has someone read to you the information sheet about this study? ☐

2) Have you had an opportunity to ask questions and discuss this study, and are you happy with the answers to all your questions? ☐

3) Do you understand that you are free to withdraw your child from this study at any time? ☐

4) Do you agree to let your child take part in this study? ☐

5) Do you consent to the use of your child's blood to measure thymic output (production of cells to help fight infection)? ☐

6) Do you consent to the use of your child's blood for a full blood count and to test for HIV? ☐

7) If the HIV test is positive, do you consent to you and your child being referred to the local family HIV services? ☐

8) Do you consent to the use of your child's medical notes alongside these tests? ☐

9) Do you understand that the information produced from these tests in this study will be anonymous, and not reveal the identity of you or your child? ☐

Name of Parent/Legal guardian _____Signature_____ Date _____

Name of Witness (if applicable)_____Signature _____Date _____

Person obtaining consent: I am satisfied that the parent/legal guardian understands what the study is about and that his/her questions have been answered.

Name _____ Signature_____ Date _____

APPENDIX III: Structure and Activities of the Child Wellness Clinic

- **Introduction** and overview of what will happen in the clinic
- **Basic details:** Name, date of birth, address, phone number, next of kin
- **Consent:** in native language, written information will be provided. A copy of the consent form will be given to the parent/guardian, and one will be kept onsite at the clinic. Please see Appendix I.
- **Medical history:** antenatal details, neonatal, family and social history, and history of medical problems including any current complaint.
- **Review of the "Road to Health" booklet:** review of record keeping, previous problems and growth.
- **Anthropometry:** weight, height, head circumference (added to the "Road to Health" booklet).
- **Review of vaccinations:** from "Road to Health" booklet and make arrangements for vaccinations if not up-to-date.
- **Physical examination:** routine infant examination performed by a paediatrician (Helen Payne, the principal investigator of this study)
- **Management:** address any arising health issues and health education
- **Phlebotomy:** A 5ml sample of blood would be taken from each child for full blood count, HIV rapid antibody test and a sample for assessment of normal parameters of immune cells (T-cell and B-cell markers). A dried blood spot for HIV DNA PCR would also be taken should any positive HIV-antibody tests arise, and if so, the child and mother would be referred to the Wesbank family HIV service.
- **Waiting for results:** The parent/guardian and infant would be asked to wait in the waiting area (estimated 20 minutes) for the results of the HIV-antibody test
- **Discussion of results:** of HIV-antibody test and referral if necessary to the local family HIV service
- **Provision:** of nutritional supplements and travel reimbursement
- **Summary:** of clinic and thanks, and reminder to return in one week for FBC results and review.

APPENDIX IV: Proforma for the Child Wellness Clinic

Name:				CWC No:	
Next of kin:				Date:	
Maternal facility No:		DOB:		Infant facility No:	
Address:				DOB:	
Phone No:				Ethnicity:	B / C / W
Consented?	Y / N			Sex:	M / F

HISTORY:

Antenatal:	Antenatal screening HIV negative in records? YES / NO; Verbal negative? Y / N Normal pregnancy? YES / NO Born on time? YES / NO If early, how early?
Neonatal:	If mum positive, clinic DNA PCR records confirm baby negative? YES / NO / NA Baby stayed in hospital after birth / during 1st month of life? YES / NO
Medical Hx:	Hospital admissions? YES / NO Medications? YES / NO Unwell in last month? YES / NO
Current problems?	Cough? YES / NO Fever? YES / NO Diarrhoea? YES / NO Vomiting? YES / NO Skin rash? YES / NO Any concerns/ worries? YES / NO
Family Hx:	TB contacts at home? YES / NO
Social Hx:	Any problems with alcohol, drugs, violence in the family? YES / NO
Feeding Hx:	In first 6mths - Exclusive breast-feeding / only formula / mixed / solids introduced early?

GROWTH: (plot on "Road to Health" booklet)

	Height / length:	Weight:	Head circumference:	Growing well
Measure:				Not growing well
Plotted z-score:				Severe Malnutrition

REVIEW VACCINATIONS (tick if already given):

Birth	BCG		OPV							
6 weeks	PCV		OPV		DTaP-IPV/HIb		Hep B		RV	
10 weeks					DTaP-IPV/HIb		Hep B			
14 weeks	PCV				DTaP-IPV/HIb		Hep B		RV	
9 months	PCV		Measles							
18 months			Measles		DTaP-IPV/HIb					
Comment / action required?										
Date of last vaccination (and which if not consistent with the above):										

EXAMINATION:

Normal vital signs? YES / NO
Heart sounds normal? YES / NO
Breath sounds normal? YES / NO
Abdomen normal? YES / NO

Skin rashes? YES / NO
Lymphadenopathy? YES / NO

BLOOD TESTS TAKEN (tick if performed):

Test:	FBC		HIV antibody		Thymic output	

Result:

MANAGEMENT:

INCENTIVE:

Nutritional supplements:	….. x Yabatwana (porridge); …. x Imunut (peanut butter)
Travel reimbursement:	R50 Shoprite voucher – YES / NO

APPENDIX V: Coded Information recorded from Child Wellness Clinic

Column headings:

CWC.No	Child wellness clinic number (chronological in order of attendance)
Initials	Child's initials as used on FBC request form
Mat Facility No	Maternal facility number to identify mother from NHLS datawarehouse
Mat.DOB	Maternal date of birth
Infant Facility No	Infant facility number
DOB	Date of birth
Sample.date	Date of sample
Ethnicity	Black, Coloured, Asian, White (B, C, A or W)
Sex	Male or Female (M or F)
Dec.age	Decimal age at the time of sample being taken
Age.wks	Age in decimal weeks at the time of sample being taken
Age.cat	Age category
Mat.stat	Maternal HIV status checked in notes and verbally (0=negative, 1=positive)
NHLS.data	NHLS confirmed maternal serostatus at child's birth (0=negative, 1=positive)
Baby.pcr	Infant's HIV proviral DNA PCR result (0=negative, 1=positive)
Ant.event	Antenatal event requiring admission to hospital (0=no, 1=yes)
Ant.info	Free text on reason for hospital admission (NAP = not applicable)
Neo.event	Neonatal event requiring admission to hospital (0=no, 1=yes)
Neo.info	Free text on reason for hospital admission (NAP = not applicable)
Med.hx	Past medical event requiring admission to hospital (0=no, 1=yes)
Med.hx info	Free text on reason for hospital admission (NAP = not applicable)
Recent.ill.mth	Recent infection within last month (0=no, 1-yes)
Unwell	Current illness limiting participation in study (0=no, 1=yes)
Fam.hx	Single parent or family support (0=support, 1=single parent and no support)
Soc.hx	Social problems at home: drugs, excess alcohol, violence? (0=no, 1=yes)
Feed	Type of feed during first 6mths? (0=breast feed, 1=bottle, 2=mixed)
Length	Length in centimetres
Wt	Weight in kilograms
HC	Head circumference in centimetres
Nut.stat	Nutritional status (0=growing well, 1=not growing well, 2=malnourished)
z.score.wt	How many standard deviations from median/mean? (0=median, 1, 2 or 3)
Im.utd	Are the immunisations up to date? (0=yes, 1=no, V=verbal report)
Baby.ab	Baby's HIV antibody status (0=negative, 1=positive)
BARC No	BARC bar code number on blood samples
Hb	Haemoglobin in g/dL
Hct	Haematocrit in L/L
Plts	Platelets per 10^9/L
Wbc	White blood cells per 10^9/L
Nt	Neutrophils per 10^9/L
Lym	Lymphocytes per per 10^9/L
Mono	Monocytes per 10^9/L
Comment	Comment on the blood test results

APPENDIX VI: Ethical and regulatory approvals

The following approvals were obtained:

- HREC Stellenbosch University
- HREC Witwatersrand University
- City of Cape Town, Department of Health
- Tygerberg Hospital Management

HREC Stellenbosch University:

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Approval Notice
New Application

Thursday, May 10, 2012
Payne, Helen H

Ethics Reference #: M12/01/005
Title: "The influence of human immunodeficiency virus and antiretroviral therapy on thymic output in infants and children."

Dear Helen Payne,

The **New Application** received on **03-Feb-2012**, was reviewed by Health Research Ethics Committee 2 via Committee Review procedures on **10-May-2012** and has been approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **10-May-2012 - 10-May-2013**

Present Committee Members:
Bardien-Kruger, Soraya S
Blaauw, Renee R
Claassens, Mareli MM
Davids, Mertrude MA
DeRoubaix, Malcolm JAM
Edwards, C E
Engelbrecht, Susan S
Fernandez, Pedro PW
Hagemeister, Dirk D
Kruger, Mariana M
Lochner, Christine C
Rosenkranz, Bernd B
Verster, Gerrit Christiaan

Please remember to use your **protocol number** (**M12/01/005**) on any documents or correspondence with the REC concerning your research protocol.

Please note that the REC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

HREC Witwatersrand University:

University of the Witwatersrand, Johannesburg

Human Research Ethics Committee: (Medical)
FWA Registered No IRB 00001223

SECRETARIAT: Suite 189, Private Bag x2600, Houghton 2041, South Africa Tel: +27-11-274 9200 Fax: +27-11-274 9261

17 May 2012 **FAXED**

Ms C Conradie

Research Operations Coordinator
Perinatal HIV Research Unit
P O Box 114
Diepkloof
1864

Fax: 086 536 7274/2855802

Dear Ms Conradie,

PROTOCOL: CIPRA - SA PROJECT 2 - CIPRA - SA PROJECT 2 - A PHASE III, RANDOMIZED, OPEN-LABEL TRIAL TO EVALUATE STRATEGIES FOR PROVIDING ANTIRETROVIRAL THERAPY TO INFANTS SHORTLY AFTER PRIMARY INFECTION IN A RESOURCE POOR SETTING "CHILDREN WITH HIV EARLY ANTIRETROVIRAL THERAPY (CHER)" - DAIDS RISK/BENEFIT CATEGORY: 45 CFR 46.406

ETHICS REFERENCE NO: 040703 **DAIDS Risk/Benefit Category: 45 CFR 46.406**

RE : APPROVAL FOR REQUEST: FOR USE OF STORED SAMPLES

We acknowledge receipt of your letter dated 15 May 2012 with the following documentation pertaining to the above-captioned trial.

The following has been approved:

* Use of stored specimens from the above-mentioned trial for a project that would fall under the scope of projects indicated in the storage consent:
- Storage Amendment Application Form dated 7 May 2012
- Motivation Letter from Dr Helen Payne dated 26 April 2012
- Proposal, "The influence of human immunodeficiency virus and anti-retroviral therapy on thymic output in infants and children, Version 1.3 dated 28 March 2012"

The above has been noted for the Ethics Committee information and records.

KINDLY FORWARD TO THE RELEVANT INVESTIGATORS / CRA / SPONSOR / STUDY CO-ORDINATORS - WHERE APPLICABLE

Regards,

PROF PETER CLEATON-JONES
For and on behalf of the Human Research Ethics Committee: (Medical)

City of Cape Town, Department of Health:

CITY OF CAPE TOWN | ISIXEKO SASEKAPA | STAD KAAPSTAD

Civic Centre
12 Hertzog Boulevard
Cape Town 8001
P O Box 2815, Cape Town 8000
Ask for: Dr G H Visser
Tel: 021 400-3981
Cell: 083 298 8718
Fax: 021 421-4894
E-mail: helene.visser@capetown.gov.za
Website: http://www.capetown.gov.za
Ref:
Filename: G:\Research\2012\HPayne10301.docx

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Kaapstad 8001
Posbus 2815, Kaapstad 8000
Vra vir: Dr G H Visser
Tel: 021 400-3981
Sel: 083 298 8718
Faks: 021 421-4894

CITY HEALTH — Specialised Health

2012-05-25

re: Research Request: The influence of HIV and ART on thymic output in infants and young children (ID NO: 10301)

Dear Dr Payne

Permission has been granted to do your research as per your protocol at the following City Health Clinic:

Eastern Sub District: Wesbank Clinic
Contact People Dr P Nkurunziza (Sub District Manager)
Tel: (021) 850-4315 / 084 800 0644
Mrs N Mgqweto (Head: PHC & Programmes)
Tel: (021) 850-4312 / 084 222 1487

Please note the following:

1. All individual patient information obtained must be kept confidential.
2. Access to the clinic and its patients must be arranged with the relevant Manager such that normal activities are not disrupted.
3. A copy of the final report must be sent to the City Health Head Office, P O Box 2815 Cape Town 8001, within 3 months of its completion and feedback must also be given to the clinics involved.
4. Your project has been given an ID Number (10301). Please use this in any future correspondence with us.

Thank you for your co-operation and please contact me if you require any further information or assistance.

Yours sincerely

DR G H VISSER
MANAGER: SPECIALISED HEALTH

cc. Dr Nkurunziza & Ms Mgqweto
Dr K Jennings
Ms Caldwell
Prof M Cotton (KIDCRU)

THIS CITY WORKS FOR YOU ESI SIXEKO SISEBENZELA WENA HIERDIE STAD WERK VIR JOU

Tygerberg Hospital Management:

Western Cape Government
Health

TygerbergHospital and
MitchellsPlain&Tygerberg Oral Health Centres
REFERENCE: Researches
ENQUIRIES: Dr M A Mukosi
TELEPHONE: 021 938-5966

ETHICS NO: M12/01/005

The influence of human immunodeficiency virus and antiretroviral therapy on thymic output in infants and children.

Dear Dr Payne

PERMISSION TO CONDUCT YOUR RESEARCH AT TYGERBERG HOSPITAL

In accordance with the Provincial Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the above-mentioned research here at Tygerberg Hospital.

Dr P E Ciapparelli
(021) 938 5883
Director

DR D ERASMUS
CHIEF DIRECTOR: TYGERBERG HOSPITAL

03.07.2012

APPENDIX VII: Ethical approval for CHER PLUS thymic output substudy

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Ethics Letter

24-Jan-2013

Ethics Reference #: M11/10/042
Clinical Trial Reference #: IR01 HD071664-01
Title: IR01 HD071664-01 "Longitudinal Neuro imaging and cognitive study of HIV-infected children"

Dear Dr Barbara Laughton,

Your letter dated 21 January 2013 refers.

The chairperson of the Health Research Ethics Committee approved the following addendum to the CHER PLUS study:

Additional 2 mls of blood to be drawn at routine yearly blood draw for the purpose of measuring thymic output.
Protocol Version 1.0 dated 20 December 2012
Information Leaflet and Informed Consent Form Version 1.0 dated 20 December 2012
Principal Investigator: Dr Helen Payne

If you have any queries or need further assistance, please contact the HREC Office 0219389657.

Sincerely,

REC Coordinator
Franklin Weber
Health Research Ethics Committee 1

APPENDIX VIII: Information / consent form for thymic output in CHER PLUS

CHER PLUS PATIENT INFORMATION LEAFLET AND INFORMED CONSENT FORM: ADDITIONAL BLOOD TO BE TAKEN AT THE ROUTINE YEARLY BLOOD DRAW

Centre: Children's Infectious Diseases Clinical Research Unit, Tygerberg Hospital
HREC study number: M11/10/042
Principal Investigator of CHER PLUS: Dr Barbara Laughton
Investigator to do thymic output analysis: Dr Helen Payne
Sponsored by: The Wellcome Trust

TITLE OF THE STUDY: THYMIC OUTPUT IN CHER PLUS

This information is provided to help you decide whether to take part in this clinical research study. Please read this form carefully and ask the study doctor or the study staff to explain any words or procedures you do not understand.

Dear parent or guardian,
Hello, my name is Helen Payne and I am a children's doctor and research fellow at Tygerberg Hospital. I am working with the CHER PLUS team and would like to invite your child to take part in a research project that will help us understand more about the way in which HIV affects the immune system of children. A better understanding will mean we, as doctors, will be able to improve the care we give to your children and help them have a healthier life.

Your child will not need any more blood tests than usual. I am only asking for your consent to take 2mls (less than half a teaspoon) extra blood at your child's routine yearly blood test. It will not mean an extra needle and 2mls more of blood will not make a difference to your child's body at all.

The following sheets explain the project in more detail and are for you to keep. When you have read it, or someone has read it to you, I or one of my colleagues, will be pleased to answer any questions you have. If you are happy to take part in the study then all you need to do is sign the consent form. No information from the study will reveal your or your child's identity, and you can withdraw from the study at any time.

I thank you in advance for your time and help.
Yours sincerely,

Dr Helen Payne

WHAT IS THE STUDY AND WHY ARE WE DOING IT?

HIV infection in children is still a major problem in South Africa. Although treatment with medicines against HIV (HAART) has saved many lives, there are still important questions we do not know the answers to. We know we should start HAART as soon as possible after birth in children who are HIV-infected; however, we do not know exactly how long we should continue HAART and when it is safe to have a treatment break. The results this study intends to produce may provide information that will help us answer these questions. This information could be important for HIV management worldwide.

WHY WILL THIS BE USEFUL?

HIV weakens the immune system and makes children vulnerable to serious infections. The immune system is controlled by a gland called the thymus. However it is not understood how the thymus reacts to HIV-infection or treatment. In this study we will investigate how HIV and HAART affect this gland and the production of immune cells (called thymic output).

WHAT DOES MY CHILD HAVE TO DO IF I AGREE TO THIS STUDY?

Your child does not have to do anything at all. During CHER PLUS, your child will have a yearly routine blood test and at the time of this blood test we would like to draw an extra 2mls of blood. This would not mean an additional needle and 2mls of blood would not make a difference to your child's body at all. I will use this blood to measure the production of immune cells from the thymus (thymic output).

YOUR CHILD'S PARTICIPATION IS VOLUNTARY

You can refuse to consent for the extra 2mls of your child's blood for this study. Even if you agree, you can withdraw consent at any time. If you decide not to take part, this will not affect the standard of care your child receives. Approximately 100 children as part of CHER PLUS will be asked to participate in this study.

WHAT ABOUT CONFIDENTIALITY?

Information about your HIV status, your child's HIV status, your child's care in the clinic and his/her blood test results will be stored in locked cabinets. All information collected during the study will be treated as confidential. There will be no difference from the way your medical information is used than it has been so far with the CHER trial and CHER PLUS. All information that is used for research or publication purposes will be kept confidential and will not have your or your child's name recorded on it. The Health Research Ethics Committee may check that these records are kept correctly.

WHAT ARE THE RISKS TO MY CHILD?

There are no serious risks to your child from this study. During the collection of blood samples, your child may experience light-headedness, pain, bleeding and/or bruising at the site on the arm where blood is taken. Blood clots may form and infections may occur, but this is unusual. Fainting may occur during or shortly after having blood drawn.

WHAT ARE THE COSTS TO ME? AND WILL I RECEIVE ANY PAYMENT FOR MY CHILD'S PARTICIPATION?

There is no cost to you for the tests. You will not receive any additional payment for your child's participation in the study.

WHAT DO I DO IF I HAVE QUESTIONS OR PROBLEMS?

Thank you for taking the time to consider this study. If at any time you have any questions during the study, please do not hesitate to contact myself (Helen Payne) or any of my colleagues.

CONTACT DETAILS:

Children's Infectious Diseases Clinical Research Unit (KID-CRU)
Ward J8, University of Stellenbosch, Tygerberg Children's Hospital
Francie van Zijl Avenue, Parow,
7505, Cape Town
hpayne@sun.ac.za
Tel: 0833 633 057

If you would like any information regarding your child's rights as a research participant, or have complaints regarding this research study, you may contact:

The Chairperson
Health Research Ethics Committee
University of Stellenbosch
Tel: (021) 938-9075

This independent committee has been established to help protect the rights of research participants and it gave written approval for the study protocol.

CHER PLUS THYMIC OUTPUT STUDY: INFORMED CONSENT SIGNATURE PAGE

Name of participant: _____

Date of Birth:_____ CHER participant number:_____

To parents/legal guardians, please TICK the box to answer YES or NO:

	YES	NO
1) Have you read or has someone read to you the information sheet about this study?	☐	☐
2) Have you had an opportunity to ask questions and discuss this study, and are you happy with the answers to all your questions?	☐	☐
3) Do you know that you are free to withdraw your child from this study at any time?	☐	☐
4) Do you agree to let your child take part in this study?	☐	☐
5) Do you consent to the use of your child's blood to measure thymic output (the production of immune cells to help fight infection)?	☐	☐
6) Do you consent to the use of your child's medical notes alongside these tests?	☐	☐
7) Do you understand that the information produced from these tests in this study will be anonymous, and not reveal the identity of you or your child?	☐	☐

Parent/Legal guardian:

_____	_____	_____	☐☐☐☐
Name	Signature	Date	Time

Witness (if applicable):

_____	_____	_____	☐☐☐☐
Name	Signature	Date	Time

Person obtaining consent: I am satisfied that the parent/legal guardian understands what the study is about and that his/her questions have been answered.

_____	_____	_____	☐☐☐☐
Name	Signature	Date	Time

APPENDIX IX: Standard operating procedure for PBMC isolation

Preparation:

1) Heat waterbath to 37°C.
2) Warm RPMI and 2 x 50ml falcons of RPMI with 2% FCS in waterbath for at least 10 mins.
3) Set up hood.
4) Check initials, DOB, IWC number of samples and place in ascending order in blue rack. Blood quantity will be between 1-2mls each.
5) Take 2 x 15ml tube per sample and label according to IWC number (one tube will be for lymphoprep, one for aspiration of PBMCs).
6) Fill 15ml vial with 3ml lymphoprep.

PBMC Isolation procedure:

1) Add 1ml RPMI to each EDTA tube of blood.
2) Using 1ml pipettes gently layer blood onto lymphoprep.
3) Note on the counting log an estimate of the volume of blood per sample.
4) Centrifuge at 2200rpm (4 acceleration, 0 deceleration) for 25 minutes.
5) First remove plasma layer to 2-3mm above layer of PBMCs and place in 1.5ml sterile eppendorf.
6) Aspirate layer of PBMCs and all lymphoprep to 2-3mm layer above RBCs.
7) Add 5ml RPMI with 2% FCS to each aliquot of PBMCs.
8) Please note whether PBMCs have >50% RBCs – these samples will need to be lysed.
9) Centrifuge 1500rpm (9 acceleration, 9 deceleration) for 10 mins.
10) Refill 50ml falcon with RPMI and 2% FCS and place in waterbath.
11) While spinning:

    Prepare cryovials and labels. Please label as follows:

    e.g. HP IWC182
    14-11-12
    PBMC

    Or… HP IWC182
    14-11-12
    FFP

12) Discard RPMI mix and add 5ml further RPMI with 5% FCS
13) Centrifuge 1500rpm (9 up, 9 down) for 10 mins.
14) While spinning:
    a. Prepare counting wells with 10ul trypan blue and haemocytometers.

b. Prepare freezing media: 90% FCS and 10% DMSO. Keep wrapped in foil and in the fridge. Discard any leftover freezing media.

15) Note: IF sample had >50% RBCs do not mix with RPMI/FCS – mix with 2ml FACS Lyse and leave in the dark for 10 mins while the remaining samples are being centrifuged. After these 10 mins, add 10ml RMPI with FCS to falcon and centrifuge for 5mins.
16) Discard RPMI mix and add 5ml further RPMI with 5% FCS
17) Mix well with 1ml pipette.
18) Then add 10ul of cell-mix to trypan blue – mixing well, then apply to haemocytometer.
19) Centrifuge again at 1500rpm for 5 minutes.
20) Count all cells in 25 large squares – note on counting log.
21) Multiply cell count _____ by 5.2ml x 2 x 10,000 to get total number of cells.
22) Calculate freezing media required to freeze each sample at a concentration of 1 x 10^7 per ml, rounded to the nearest 100ul.
23) Discard RPMI mix.
24) Aspirate remaining RPMI and discard.
25) Mix required quantity of freezing media with cell pellet and add to cryovial.
26) Place in Mr Frosty in -80°C freezer. Please ensure that isopropranol is at the level of 250ml (if lower, top up). Change Mr Frosty after every fifth use.
27) Place plasma in plasma freezer box at -80°C.
28) Remove samples frozen in Mr Frosty after 24-48hrs and place in liquid nitrogen. Defrost Mr Frosty ready for next session.

APPENDIX X: Standard operating procedure for measuring thymic output

Thymic Output SOP:

Isolation of naïve CD4 T-cells and flowcytometry cell-preparation

October 2013

CONTENTS:

APPENDICES:

1) Abbreviations

CHER	Children with Early antiretroviral therapy trial
cRPMI	Complete RPMI media (with 2% FCS)
FACS	Fluorescence activated cell-sorting (but used to indicate standard flowcytometry in this text)
FACS buffer	Buffer used to prepare flowcytometry specimens
FCS	Fetal calf serum
FOXP3 buffers	Fixative and permeabilisation buffers
CWC	Child Wellness Clinic (healthy controls)
LD	Live-dead viability dye for flowcytometry
MACS	Miltenyi biotec magnetic beads isolation procedure
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
TRECs	T-cell receptor excision circles

2) Background of this approach to measuring thymic output

The major limitation of many surrogate markers used for estimating thymic output is that these single measures (usually taken from peripheral blood) do not take into account rates of peripheral T-cell division and death. Thereby these single measures cannot be reliable due to the dilution effect of cell-division - something which is especially relevant in HIV-infected individuals in whom increased rates of cell division and CD4 T-cell death is well recognized.

TRECs (T-cell receptor excision circles) are one of these surrogate markers of thymic output. TRECs are episomal DNA that exist within a recent thymic emigrant and indicate that gene rearrangement within the thymus has occurred. The do not divide during mitosis and therefore they become rapidly diluted in the peripheral blood in conditions of increased cell proliferation.

This SOP describes the procedure to estimate thymic output that attempts to overcome this limitation by combining rates of proliferation of naïve CD4 T-cells and TRECs from purified naïve CD4 T-cells. These measures are combined in a mathematical model that also takes into account total blood volume and peripheral naïve CD4 T-cell count. Proliferation of naïve CD4 T-cells is measured by flowcytometry (delineated by the markers CD3+CD4+CD45RA+Ki67+). Naïve CD4 T-cells are purified by magnetic bead isolation and DNA PCR is used to quantify TRECs.

3) Overview of procedure

Morning (~5hrs)
1. Thawing cryopreserved PBMCs.
2. Dividing the PBMCs into three aliquots: a. Aliquot to be used for DNA extraction (leave for afternoon) b. To be used for magnetic bead isolation of naïve CD4 T-cells c. To be used for flowcytometry
3. To save time aliquot (b) and (c) are processed as two separate procedures in parallel, i.e. during the incubation phases of the magnetic bead isolation, fluorochrome-conjugated antibodies are prepared and mixed with PBMCs etc. (When finished, I will collect and run the prepared PBMCs on the canto)
Afternoon (~2hrs)
4. DNA extraction: a. PBMCs from the morning b. Purified naïve CD4 T-cells from magnetic bead isolation
5. Nanodrop the 24 specimens of extracted DNA (if time)

4) Schedules, worksheets and specimen records

Between the three of you there should be 50-60 days work and I can offer to pay R900 per day (7 hours work for 12 samples); and I will pay you for the days of training as well. I hope this will be okay. If it is acceptable to you, I will pay you at the end of each month for the number of days you have done.

I have printed out a calendar that I will keep on my desk so you may put your name down for the days that you would like to do. So we may all plan our working weeks, I would like ask if you could put your name down for the days you would like to do by the Friday of the week before (but of course you can mark you name down as far in advance as you wish). When you mark your name down you can collect a pack of 2 worksheets and list of specimens required (as per appendices).

Appendix A is a worksheet that should be used on a daily basis. It contains an overview of the procedure and table to record the cell count and aliquot details, plus space to comment on anything unusual about the specimen. Appendix B is an example of a list of 12 samples that you will receive each day including specimen identification details, storage location and identification codes. Appendix C is the worksheet required for DNA extraction.

Please return the 3-page pack to my desk after the experiment is finished, thank you.

5) Reagents, consumables, storage and equipment

Consumable	Supplier	Cat no.	Location	Comments
THAWING PBMCS:				
cRPMI	Sigma-Aldrich	R4130-10L	Fridge	1L dH2O, 16.9g RPMI, 2% FCS
Fetal bovine serum	PAA laboratories	A15-101	Immunofreezer 4, tray 3	50ml aliquot in fridge
MACS ISOLATION:				
Naïve CD4+ T-cell isolation Kit II	Miltenyi Biotec	130094131	Fridge	
LS columns (and pre-separation filters)	Miltenyi Biotec	130042401	My cupboard	
MACS BUFFER	Made in lab	NA	Fridge	1L PBS, 2mls EDTA, 5g BSA
FLOWCYTOMETRY:				
Ki67 PE	BD Pharmingen	556027	Fridge	
CD4 PERCP	Invitrogen	MHCD0431	Fridge	
CD3 FITC	Insight Biotech	300306	Fridge	
CD45RA FITC	Invitrogen	MHCD45RA 01	Fridge	
CD3 PE	Invitrogen	CD0304	Fridge	
FoxP3 Fix/Perm	eBioscience	00-5521-00	Fridge	
Permeabilising buffer	eBioscience	00-8333-56	Fridge	
Fixable Viability Dye eFluor 660	eBioscience	65086418	Upright -80 (~LN room)	
CD19 PERCP	C. Bioscience	302228	Fridge	
CD56 PE-Cy7	Insight Biotech	304628	Fridge	
CD8 BV510	Biolegend	301048	Fridge	
IGD BV510	BioLegend	302824	Fridge	
CD27 BV421	BioLegend	348219	Fridge	
PBS (from tablets)	MP Biomedicals	92810307	My cupboard	
FACS BUFFER	Made in lab	NA	My cupboard	1L PBS, 1g BSA, 20mls 1% Na-azide
DNA EXTRACTION:				
QiAmp mini kit	Qiagen	51304	My cupboard	
Dulbecco PBS liquid	Invitrogen	14190094	My cupboard	
EQUIPMENT:				
Quadromacs magnet	Miltenyi Biotech	NA	My cupboard	
Pipettes and racks	Mixed	NA	My cupboard	
FACS tubes and caps	Lasec	352054	My cupboard	
Ice-packs	Mixed	NA	Freezer 4	
Blue cool box	Big Jim	NA	Gates TC	
Sterile eppendorfs	Mixed	NA	Above fridge	
Tips, tubes, gloves	Mixed	NA	General stock	

* Please inform me if anything is running out and I will make/find more.

*All antibodies are mouse anti-human.

6) Samples:

Thymic output will be measured from HIV-infected children on and off ART (CHER specimens) and HIV-uninfected healthy children (CWC specimens).

a. Storage and location

i. **CHER:** All CHER specimens are stored in the Immunology liquid nitrogen tank in column 3 in boxes 1 to 10 in descending order.

ii. **CWC:** CWC specimens are stored in the Immunology liquid nitrogen tank in column 4 in slots 7, 8 and 9 (in descending order).

- Slot 7: Box CWC2 = specimens no. 54 to 178
- Slot 8: Box CWC3 = specimens no. 179 to 252
- Slot 9: Box CWC4 = specimens no. 253 to 333

  NB. CWC specimens 334 to 487 are in liquid nitrogen storage in Virology. I will transfer them to slots 7, 8 and 9 when space becomes available.

b. Health risk and necessary precautions

i. **Liquid nitrogen:** Please take usual precautions with retrieving the samples from liquid nitrogen (lab coat, LN2 gloves, shoe-covers and protective eye-shield).

ii. **Caps for CHER specimens until cells are fixed:** Please keep caps on top of the FACS tubes containing CHER specimens when outside of the laminar flow cabinet especially during vortex and centrifugation until the cells are fixed with FOXP3 fixation buffer (step 15).

iii. **Spillage procedures:** In the event of a spillage of CHER specimens (live HIV-infected cells), clean contaminated equipment thoroughly with virkon and dispose in sealed rubbish bags. If contact with skin puncture or mucous membranes, immediately rinse with water then seek medical attention.

7) Procedure

Thawing cryopreserved PBMCs:

1) Switch on waterbath at 37°C. Label 12 x 15ml falcon tubes: 1 to 12, then Aliquot 3ml FCS and 2ml cRPMI into each. Plus place one 15ml falcon containing 7mls FCS to warm in the waterbath for washing cryovials to retrieve any leftover cells.

2) Warm aliquots of FCS/RPMI and 2 x 50ml falcon tubes of cRPMI in waterbath for 15mins while setting everything up.

SET-UP:

- Tip boxes (10µl, 20µl, 200µl, 1ml)
- Pipettes and multi-dispenser (tray of multi-dispenser tips)
- 96-well plate for counting, trypan blue, hand-held counter
- 4 type of racks: 50ml, 12ml, FACS tube and eppendorf racks
- Sponge floatation device for thawing specimens
- 1L jug for discarded fluids with 10mls Virkon
- Put QuadroMACS magnet in small freezer compartment
- Magnet stand and rack
- 4 x 50ml falcon tubes of MACS Buffer in freezer compartment
- 24 FACS tubes (with caps if processing CHER specimens, no need for caps with CWC specimens). Label FACS tubes: 1A, 2A, 3A etc. until 12A; 1B, 2B, 3B etc. until 12B. Please use green pen for Panel A and blue pen for Panel B. In some cases there will only be panel A – therefore only 12 FACS tubes required
- 24 sterile eppendorfs. Label: 1P, 2P, 3P etc. until 12P; 1R, 2R, 3R, etc. until 12R. Please use black pen for “P” for PBMCs and blue pen for “R” for naïve CD4 T-cells (from CD45RA abbreviated to RAs). Likewise, when no Panel B there will only be 12 eppendorfs labelled 1R to 12R (no 1P to 12P needed).
- 12 x 15ml falcon tubes. Labelled 1F, 2F, 3F etc. until 12F. “F” is for flowcytometry.
- 12 x MACS columns (± pre-separation filters for any samples with less than 2x10^6 PBMCs).
- 1L bottles of PBS and FACS buffer as will need refills
- Place ice packs (in drawer 3 immunofreezer 4) in blue cool box.
- Check fridge temperature is >4°C – if not please adjust accordingly as

antibody-staining may be inhibited if fridge is too cold.

3) Retrieve aliquot of viability stain from upright -80 freezer (keep wrapped in foil as light-sensitive), and retrieve specimens from liquid nitrogen tanks (please use usual precautions including use of tweezers to remove specimens from the box).

    a. Please check id details on each specimen 6-digit identification number, date of specimen and sample id number match specimen sheet given (as per example in Appendix B).

    b. If no cell count is recorded on specimen sheet provided, it is likely to be hand written on the vial, if this is the case please record this pre-frozen cell count in the space provided.

    c. Keep specimens in cool box with ice-packs while identifying other specimens.

    d. Finally, when all samples are retrieved from liquid nitrogen, add specimens to sponge floatation device in ascending order and place in waterbath to thaw.

4) Please observe thawing process carefully – this may take as little as 30 seconds only. While thawing quickly remove warmed FCS from waterbath, place in rack in ascending order and remove lids. As soon as the aliquot has thawed pipette sample into the 15ml falcon tube with warmed FCS and gently resuspend 5-times. Add 0.5ml warmed FCS to each empty cryovial to retrieve any leftover cells and pipette into their respective falcon tubes.

5) First centrifugation at 1500rpm for 5min at room temperature.

    a. While spinning prepare mastermixes for Panels A and B (as per quantities indicated on specimen sheet (see example in Appendix B). Please have the light off in the hood when working with fluorochrome-conjugated antibodies, and please mix with a pipette and do not vortex. NB. Viability stain (LD) and Ki67PE do not go into the mastermix, this is added separately.

    b. When spinning complete discard supernatant supernatant.

    c. Using multi-dispenser add 4ml warmed cRPMI to each cell discard supernatant and resuspend solution with 1ml pipette. NB: when using the multi-dispenser, always discharge 1ml first as the first dispensing is not always accurate.

6) Second centrifugation at 1500rpm for 5min at room temperature.

    a. While spinning prepare slides for counting, place 10µl trypan blue in 12 wells of the 96-well counting plate, refill multi-dispenser with 50ml warmed cRPMI.

    b. When spinning complete discard supernatant supernatant

c. Using multi-dispenser add 4ml warmed cRPMI to each cell discard supernatant and resuspend solution with 1ml pipette.

d. Take 10ul sample solution and mix 10-times with trypan blue then apply to haemocytometer.

e. Count all live and dead PBMCs – and place the number counted in the first 2 columns of the table on the worksheet (Appendix A).

f. Multiply the number of live PBMCs by 84,000 to get the number of live PBMCs in whole sample (see example below). This figure is derived by 2 x 4.2 x 10,000, which reflects a 2-fold dilution with trypan blue, 4.2mls of cRPMI and the magnification of the haematocytomter. 4.2mls is used because after the centrifuged sampled is has it's supernatant discarded, approximately 200µl of liquid remains in the bottom of the falcon tube.

ID	Live PBMC	Dead cells	Total live
1	48	2	4.0x10^6
2	76	24	6.4x10^6

g. Finally, refill the 50ml falcon tubes with cRPMI and return to fridge.

Dividing the PBMCs into three aliquots:

a. Aliquot to be used for DNA extraction (leave for afternoon)
b. To be used for magnetic bead isolation of naïve CD4 T-cells
c. To be used for flowcytometry

7) Divide PBMCs into 3 aliquots as per guide below and indicate in table on worksheet the quantities (see example).

- FACS aliquot should be placed in falcon tubes labeled 1F to 12F.
- PBMC DNA aliquot should be placed in eppendorfs labeled 1P to 12P. Put PBMC DNA aliquot aside for DNA extraction in the afternoon.
- MACS aliquot is the remaining solution in the original tubes labeled 1-12.

- *If total number of thawed live cells >4x10^6 – 300µl for FACS, 600µl for PBMC DNA and remaining for MACS*
- *If total number of thawed live cells <4x10^6 - use 300µl for FACS and remaining for MACS (no aliquot for DNA extraction of PBMCs)*
- *If total number of thawed live cells <2x10^6 – as above, plus use pre-separation filters to maximise yield (or if samples appear clumpy)*
- *If <1x10^6 use for FACS only (no MACS)*

ID	Live PBMC	Dead cells	Total	PBMC µl	Comment
1	48	2	4x10^6	NA	
2	76	24	6.4x10^6	600	
3	20	38	1.7x10^6	NA	Filter used

NB. Not all CHER samples will require an aliquot of PBMCs for DNA extraction. – this will be indicated on all worksheets collected daily (Appendices A, B and C), i.e. the mastermixes and DNA extraction sheet will be adjusted accordingly.

To save time aliquot (b) and (c) are processed as two separate procedures in parallel, i.e. during the incubation phases of the magnetic bead isolation, fluorochrome-conjugated antibodies are prepared and mixed with PBMCs etc. In the detailed instructions below the intracellular-staining for flowcytometry steps are written in *underlined italics* and the MACS isolation steps are written in normal script.

8) Centrifuge both MACS and flowcytometry (FACS) aliquots at 1500rpm for 5min at room temperature.

- MACS aliquot wash in existing cRPMI.
- *FACS aliquot wash with 1ml PBS. After spinning, discard supernatant and place aside.*

9) Mix MACS buffer and antibody cocktail:

   a. Aspirate MACS supernatant completely

   b. Add 40µl MACS buffer per 10^7 cells and resuspend well.

   c. Add 10µl naïve CD4 T-cell biotin-antibody cocktail per 10^7 cells. Mix really well.

   d. Incubate for 30mins in the dark in the fridge. Please note time incubation will be complete on the worksheet.

10) *Add 1ml PBS to FACS aliquots, centrifuge at 1500rpm for 5min at room temperature*

   a. *Meanwhile, aliquot 10µl of mastermix A to 12 FACS tubes for Panel A (labeled 1A to 12A).*

   b. *Then, aliquot 12µl of mastermix B to 12 FACS tubes for Panel B (labeled 1B to 12B).*

   c. *NB. Please check specimen details sheet (as per Appendix B) to confirm quantity for mastermixes and aliquots as the panel will change slightly with some of the CHER specimens (i.e. there may not always be two panels).*

   d. Please have the light off in the hood when working with fluorochrome-conjugated antibodies.

   e. *When spinning finished, discard supernatant PBS*

   f. *Add 0.5µl viability dye to each PBMC discard supernatant*

   g. *Add 100µl PBS to resuspend each PBMC discard supernatant (100µl only is sufficient as some PBS will remain in the tip of the falcon tube after discarding supernatant). If only doing Panel A – no need to add any PBS here.*

h. *Add 100µl to each respective FACS tube i.e. from falcon tube 1F add 100µl of resuspended PBMCs to FACS tube 1A and 1B.*

i. ***Apply FACS tube caps if working with CHER specimens.*

j. *Vortex for 5 seconds each*

e. *Incubate for 30mins in the dark in the fridge. Please ensure fridge temperature is >4°C and note time incubation will be complete on the worksheet.*

11) When MACS antibody-cocktail incubation complete, add 2ml MACS buffer with multi-dispenser and centrifuge at 1500rpm for 5min at 4°C. Rinse multi-dispenser with distilled H2O.

- *Meanwhile, prepare fixation and permeabilisation buffers.*
    - *Fix/Perm: for 24 tubes mix 625µl fixative-concentrate with 1875µl of diluent (1 in 4 dilution).*
    - *Perm/Wash: for 24 tubes mix 2.5ml permeabilisation-concentrate with 22.5ml distilled H2O (1 in 10 dilution).*
    - *Store in fridge until required.*
    - *NB. These buffers must be made fresh daily.*
    - *NB. If Panel B excluded, please only make half the quantities above.*

12) When MACS aliquots finishes spinning:

a. Aspirate MACS supernatant completely

b. Add 80µl MACS buffer per 10^7 cells and resuspend well

c. Add 20µl anti-biotin microbeads per 10^7 cells, mix really well.

d. Incubate for 30mins in the dark in the fridge. Please note time incubation will be complete on the worksheet.

13) When FACS aliquots incubation complete, add 3ml FACS buffer per FACS tube using multi-dispenser, and centrifuge at 1500rpm for 5min at room temperature. Rinse multi-dispenser with distilled H2O.

a. Discard supernatant FACS tubes

b. Add 100µl Fix/Perm per tube (use 100µl multi-dispenser)

c. Vortex (with caps on)

d. Incubate at room temperature for 15mins in the dark (cover completely with foil)

14) When MACS microbeads incubation complete, add 2ml MACS buffer with multi-dispenser and centrifuge at 1500rpm for 5min at 4°C.

a. Label 12 x 15ml falcon tubes 1M, 2M, 3M etc. to 12M

b. Retrieve QuadroMACS magnet from freezer shelf and attach to stand. Care not to trap fingers, as magnetic force can be strong.

c. Apply 4 columns to the magnet with place tubes 1M to 4M beneath in rack. If a pre-separation filter is required, it is placed above the columns before rinsing.

d. Rinse columns with 3ml cold MACS buffer.

e. When buffer has run through, discard eluted buffer but retain falcon tubes.

f. When centrifugation complete, discard supernatant from all 12 tubes.

g. Add 3ml MACS buffer to each tube using multi-dispenser.

h. Place tubes labeled 5 to 12 in the fridge as only 4 samples can be processed at once.

i. Using 1ml pipette, resuspend sample and apply to top of column (first 3mls). Care that bubbles are not created or applied to the column as it can obstruct the elution.

j. Add 3ml MACS buffer to the empty tubes to “mop up” any leftover cells (for the second 3mls).

k. When the column is dry following the first 3mls, apply the second 3mls. NB. If using pre-separation filter for clumpiness, please use inside of syringe to massage the filter after the first 3mls and after the second 3mls.

l. Likewise, when the column is dry following the second 3mls, apply 3mls MACS buffer from multi-dispenser directly to the column (third 3mls).

m. Finally, when the column is dry following the third 3mls, apply 3mls MACS buffer from multi-dispenser directly to the column (fourth 3mls).

n. The falcon tubes (1M to 4M) beneath the columns should then contain 12mls of diluted but purified naïve CD4 T-cells.

o. Centrifuge these purified cells at 1500rpm for 10mins at 4°C.

p. Remove the next set of 4 falcon tubes (5 to 8; or 9 to 12) from the fridge and recommence the process above from “i“ to “o”.

q. Finally refill MACS buffer 50ml aliquots when empty and keep in freezer compartment until the end of the experiment.

15) Meanwhile, when the FACS fixation incubation stage is complete:

a. *Add 0.5ml Perm/Wash per each FACS tube (use multi-dispenser)*

b. *Centrifuge at 1500rpm for 5mins at room temperature*

c. *While spinning prepare Ki67-mix: 24µl Ki67PE with 1200µl Perm/Wash for 24 tubes (please half both quantities if only using Panel A).*

d. *When centrifugation is finished, discard supernatant supernatant, then add 50µl of KI67-mix per FACS tube*

e. *Vortex (FACS caps no longer necessary as cells are now fixed)*

f. *Incubate for 30mins in the dark in the fridge*

16) While MACS purified cells are spinning, prepare to count the cells:

a. Haematocytometer ready and 10ul of trypan blue per counting well of 96-well plate.

b. When spinning complete discard supernatant supernatant

c. Add 800ul of PBS to each falcon tube.

d. Resuspend solution with 1ml pipette and apply to eppendorfs labeled 1R to 12R.

e. Take 10ul sample solution, from 1R to 12R, and mix 10-times with trypan blue then apply to haemocytometer.

f. Count all naïve CD4 T-cells – and place the number counted in the first "RAs ct" column of the table on the worksheet.

g. Multiply the number of cells by 20,000 to get the number of naïve CD4 T-cells (see example below). This figure is derived by 2 x 1 x 10,000, which reflects a 2-fold dilution with trypan blue, 1ml of PBS and the magnification of the haematocytomter. 1ml is used because after the sample is centrifuged and supernatant is discarded, approximately 200ul of liquid remains in the bottom of the falcon tube.

ID	Live PBMC	Dead cells	Total	FACS µl	PBMC µl	RAs ct	RA Total	Comment
1	48	2	4x10^6	420	NA	15	3x10^5	
2	76	24	6.4x10^6	300	600	33	6.6x10^5	

17) *When FACS Ki67-incubation is complete:*

a. *Add 0.5ml Perm/Wash per FACS tube*

b. *Centrifuge at 1500rpm for 5mins at 4°C*

c. *Discard supernatant*

d. *Add 200µl FACS buffer per tube*

e. Place in fridge – I will collect and run the PBMCs on the canto

18) Finally, when all 12 samples have had the naïve CD4 T-cell fraction isolated and counted – place the 12 eppendorfs labeled 1R to 12R along with the PBMC DNA aliquots labeled 1P to 12P. Leave at room temperature for 1-2 hours until DNA extraction. Please return any remains of viability stain aliquot to the -80 freezer. Please wash the multi-dispenser tips with distilled water and then spray with ethanol at the end of each day.

DNA extraction:

a. PBMCs from the morning
b. Purified naïve CD4 T-cells from magnetic bead isolation

19) Heat hot-block to 56°C.

20) Move eppendorf-centrifuge into Gates TC room. Centrifuge all specimens 1R-12R and 1P-12P at 4,000rpm for 4mins. While spinning set-up hood:

SET-UP:

- Tip boxes (20µl, 200µl, 1ml)
- Pipettors
- 2 type of racks: 50ml and eppendorf racks x 5
- 24 mini-spin columns (or however many extractions required)
- 24 sterile eppendorfs (or however many extractions required)
- Box of Qiagen DNA extraction kit (Qiagen protease, >95% ethanol, buffers AL, AW1, AW2 and AE)
- Dulbecco's Phosphate Buffered Saline
- Label printer

21) Discard supernatant.

22) Add 1ml Dulbecco's PBS and resuspend by vortex for 15 seconds. Please use PBS from 50ml aliquots rather than from the original 500ml bottle. Also for DNA extraction please do not use the multi-dispenser as traces of salts can have inhibiting effects on PCR.

23) Centrifuge at 4,000rpm for 4min; afterwards discard supernatant supernatant.

24) Add 20µl Qiagen protease (protease digestion), mix 10-times.

25) Add 100µl Dulbecco's PBS and 200µl buffer AL (lyse buffer).

26) Vortex for 15 seconds

27) Incubated at 56°C on the hot-block for 10mins

- Meanwhile open mini-spin columns and label 1R-12R and 1P-12P (or as per the day's experiment)
- Note PBMC or naïve CD4 T-cell count on the table on the DNA extraction worksheet (Appendix C). Naïve CD4 T-cell count can be taken straight from final column of table in MACS/FACS worksheet. To calculate PBMC number = 0.14 x total no. PBMCs (i.e. 0.14 = 0.6/4.2 = 600ul of 4.2mls of PBMCs were put aside in step 7).
- Then calculate the required elution volume: samples with <1x10^6 cells will be eluted in 50µl of AE buffer, and samples with >1x10^6 cells will be eluted in 80µl of AE buffer.

Sample code	Sample type	Cell count	Elution volume
1P	PBMC	9x10^5	50µl
2P	PBMC	1.3x10^6	80µl

28) When incubation complete, briefly centrifuge tubes to remove drops from the lid.

29) Add 200µl ethanol to each sample

30) Mix by pulse-vortex for 15 seconds

31) Briefly centrifuge tubes again – also to remove drops from the lid.

32) Apply contents to QIAmp mini spin columns. ***Please check specimen ID matched the ID written on the spin columns***

33) Centrifuge at 8000rpm for 1min.

34) Reapply filtrate to column and spin again at 8,000rpm for 1min.

- While spinning, arrange 3 rows of collection tubes (i.e. 3 tubes per sample) in eppendorf racks.

35) Transfer column onto new collection tube, add 500µl buffer AW1 to each column, and spin again at 8,000rpm for 1min. Discard old collection tubes and filtrate.

36) Transfer column onto new collection tube, add 500µl buffer AW2 to each column, and spin again at 14,000rpm for 3min. Discard old collection tubes and filtrate.

37) Transfer column onto new collection tube and spin again at 14,000rpm for 1min. Discard old collection tubes and filtrate. Note – here another buffer is not added, the extra spin step helps to remove contaminants that might inhibit PCR.

38) Transfer column onto sterile eppendorf and discard filtrate and tube.

39) Add 50µl buffer AE to all columns and incubate at room temperature for 5min.

- While incubating, print required number of labels in this format. Please refer to the specimen location list for the sample label code:
    - Sample ID/label code e.g. HP-CWC062
    - Date e.g. 04.10.13
    - Sample type e.g. PBMC DNA; or RA DNA

40) Centrifuge at 8,000rpm for 1min.

41) Add further 30µl Buffer AE to samples with >1x10^6 cells. For those samples with <1x10^6 reapply the 50µl of eluate to column – followed by 1min incubation

42) Centrifuge at 8,000rpm for 1min.

43) Discard columns and apply labels.

44) Nanodrop as per instructions in next section (if time).

45) Then store at -20°C for PCR analysis. Can leave samples in green eppendorf rack and place directly in freezer (Immuno Freezer 3, 5th drawer).

NB. When tidying up – please place waste fluid in a waste bottle under sink in Gates TC room. When waste fluid bottle is full, dispose with experiment waste bag in red biohazard rubbish container.
NB. If working with 24 specimens, DNA extraction will be split into two groups of samples and perform each step alternately i.e. while one group of samples are spinning, prepare the others with buffers etc. If you do this, it is easier to keep colour-coded samples in separate groups.

Nanodrop the 24 specimens of extracted DNA (if time)

On the days when there are around 24 specimens to extract DNA, you may not have time to do measure these samples on the Nanodrop as well – so just let me know if this is the case and I will do it. However, on the days when there are only 12 specimens there should be enough time.

46) Log into the computer your usual MED_BIOCHEM login details. Select the Nanodrop (ND-1000) icon, then “nucleic acid” analysis.

47) Use aliquots of 1.2µl for both nuclease-free water and extracted DNA.

48) Use 1.2µl of nuclease-free water to initiate the nanodrop, and then blank once with 1.2µl AE buffer before beginning.

49) Place 1.2µl of sample on the nanodrop and click on “measure”.

50) In between samples, use 1.2µl of AE buffer to blank.

51) After each sample and each blank, wipe the nanodrop with tissue paper.

52) Please note ng/ul, 260/280 and 260/230 ratios in the spaces provided on the worksheet. Record to 1 decimal place.

53) Repeat the nanodrop with 1.5µl once only if the sample readings are widely out of normal range.

54) When complete, store extracted DNA at -20°C for PCR analysis. Can leave samples in green eppendorf rack and place directly in freezer (Immuno Freezer 3, 5th drawer).

8) Training

I would suggest 3 days of training (or more if you prefer): one day observing the procedure, and two days performing the technique under observation (starting with 4 samples/day, increasing to 8 and then 12 when ready). On the final day the purity of the naïve CD4 T-cells isolation will be checked (ideally >95%).

SOP APPENDICES:

A) MACS/FACS worksheet
B) Example of specimen details, storage location and identification codes
C) DNA extraction worksheet

SOP APPENDIX A:

H Payne: Thymic output in paediatric HIV

MACS/FACS, worksheet no:…….. Date of procedure:……..... Performed by:……

1) Aliquot 3ml FCS and 2ml RPMI into 12x 15ml falcon tubes
2) Warm FCS and cRPMI in waterbath for 15mins at 37°C while setting everything up
3) Retrieve specimens (keep in cool box with ice-packs while identifying) then place in waterbath
4) As soon as thawed pipette into 15ml falcon tube with warmed FCS
5) Centrifuge 1500rpm for 5min, Discard supernatant, add 4ml cRPMI (Prepare mastermixes A and B)
6) Centrifuge 1500rpm for 5min, Resuspend in 4ml cRPMI (prepare for counting & tubes for division)
7) Count and divide cells (place PBMC aliquot aside for the afternoon)

ID	Live PBMC	Dead cells	Total live	PBMC?	RAs ct	RA Total	Comment
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							

MACS Isolation:	**Flowcytometry Preparation:**
Wash in existing cRPMI, 1500rpm, 5min	Wash with 1ml PBS: 1500rpm, 5min, discard supernatant
Aspirate supernatant, add **40µl** MACS buffer up to 10^7 PBMCs + **Antibody cocktail 10µl; MIX WELL** INCUBATE (30min in dark/fridge).	
Time incubation complete:_____	Wash with 1ml PBS: 1500rpm, 5min, discard supernatant - Aliquot 10µl mastermix A per FACS tube - Aliquot 12µl mastermix B per FACS tube Add LD stain to tubes (0.5µl of LD stain per tube) Resuspend with 100µl PBS, then add to FACS tubes Vortex; INCUBATE (30min in dark/fridge).
WASH with 2ml MACS buffer, 1500rpm, 5min	Prepare FOXP3 buffers: - Fix/Perm = 625µl fix-conc + 1875µl diluent - Perm/Wash = 2.5ml perm-conc + 22.5ml H2O
Discard supernatant, add **80µl** MACS buffer **+ 20µl Anti-Biotin Microbeads; MIX REALLY WELL** INCUBATE (30min in dark/fridge).	Time incubation complete:_____
Time incubation complete:_____	Wash with 3ml FACS buffer 1500rpm, 5min, discard supernatant **Add Fix/Perm:** 100µl Fix/Perm per tube,

	Vortex INCUBATE (15min room temp/dark). Time incubation complete:_____
WASH 2mls MACS buffer, 1500rpm, 5min, discard supernatant **MACS MATRIX COLUMN:** Rinse with 3ml cold MACS Buffer, discard Resuspend sample in 3ml MACS buffer, apply to column and repeat Apply 2 x 3ml MACS buffer to columns, WASH for 10mins, 1500rpm, discard supernatant, resuspend in 800µl PBS, transfer to eppendorf, COUNT	WASH - 0.5ml Perm/Wash, 1500rpm, 5min, discard supernatant Prepare 24µl Ki67PE + 1200µl Perm/Wash Add 50ul per FACS tube, vortex INCUBATE (30min in dark/fridge). Time incubation complete:_____ Wash with Perm/Wash, 1800rpm, 5min, discard supernatant Add 200µl FACS buffer per tube, place in fridge

[1]

[1] If total no. thawed cells >4x10^6 – 300ul for FACS, 600ul for PBMC discard supernatant and remaining for MACS
If <4x10^6 cells, use 300ul for FACS and remaining for MACS (no PBMCs)
****If <2x10^6 = Use pre-separation filters**, If <1x10^6 PBMCs for FACS only**

THYMIC OUTPUT SOP APPENDIX B: SAMPLE IDENTIFICATION EXAMPLE

Box Location	Box No.	Box position	Study ID	Visit date	Specimen ID	Cell count	Label code	Exp. code	Comment
LN column 3	2	4,003	136236	25-Oct-06	CP0605B40740	7.4e^6CEL	CHER-0120	1	
LN column 3	2	4,004	132152	26-Oct-06	CP0605B36151	5.1e^6CEL	CHER-0086	2	
LN column 3	2	4,005	149366	26-Oct-06	CP0605B40723	9.0e^6CEL	CHER-0115	3	
LN column 3	2	4,007	148846	26-Oct-06	CP0605B40829	7.3e^6CEL	CHER-0130	4	
LN column 3	2	4,008	147636	27-Oct-06	CP0605B40645	4.4e^6CEL	CHER-0112	5	
LN column 3	2	4,009	149546	30-Oct-06	CP0605B40711	7.1e^6CEL	CHER-0113	6	
LN column 3	2	4,010	149726	30-Oct-06	CP0605B40726	7.6e^6CEL	CHER-0116	7	
LN column 3	2	5,004	144036	02-Nov-06	CP0605B40767	1.7e^6CEL	CHER-0124	8	
LN column 3	2	5,007	150556	16-Nov-06	CP0605B40738	1.4e^6CEL	CHER-0119	9	
LN column 3	2	5,009	136646	18-Dec-06	CP0605B40201	8.4e^6CEL	CHER-0104	10	
LN column 3	2	6,002	145316	22-Nov-06	CP0605B40778	7.3e^6CEL	CHER-0125	11	
LN column 3	2	6,003	138626	25-Jan-07	CP0605B35820	9.6e^6CEL	CHER-0082	12	

PREPARATION OF FLOW-CYTOMETRY DYES: (24 tubes altogether) 2 x panel per sample (A+B)

Panel A	Vol/sample	Mastermix	Panel B	Vol/sample	Mastermix
CD3PECy7	1	12	CD56PECy7	1	12
CD4PERCP	4	48	CD19PERCP	1	12
Ki67PE	1	NA	Ki67PE	1	NA
CD45RAFITC	2	24	CD3FITC	1	12
Viability	0.5	NA	Viability	0.5	NA
HLA-DR ef450	1	12	CD27 BV421	5	60
CD8 BV510	2	24	IgD BV510	1	12
Volume per FACS tube		**10µl**	**Volume per FACS tube**		**9µl**

Sample identification, sheet no:…….. Date of procedure:…… Performed by:…….

SOP APPENDIX C: DNA Extraction

1) Set up hood, heat hot-block to 56°C
2) Spin 4,000rpm 4mins, discard supernatant supernatant
3) Add 1ml PBS, vortex 10 seconds: spin samples 4,000rpm, 4min, discard supernatant
4) Add 20µl QIAGEN Protease, mix well
5) Add 100µl PBS
6) Add 200µl Buffer AL
7) Mix by pulse-vortex for 15secs
8) Incubate at 56°C for 10mins (meanwhile calculate elution volumes and label spin columns)
9) Briefly centrifuge to remove drops from lid
10) Add 200µl ethanol
11) Mix by pulse-vortex for 15secs
12) Briefly centrifuge again to remove drops from lid
13) Apply contents to QIAmp Mini spin column in a 2ml collection tube
14) Spin 8,000rpm for 1min
15) **Reapply filtrate** to column; Spin 8,000rpm for 1min
16) Discard filtrate and tube; new tube, add **500µl AW1**; Spin 8,000rpm for 1min
17) Discard filtrate and tube; new tube, add **500µl AW2**; Spin 14,000rpm for 3mins (print labels)
18) Discard filtrate and tube; new tube, spin 14,000rpm for 1min
19) Discard filtrate and tube, place column in **microcentrifuge tube**
20) Add 50µl Buffer AE to column, incubate for 5mins at room temperature, 1min spin 8,000rpm
21) Add further 30µl Buffer AE to samples >1x10^6 cells; if <1x10^6 reapply 50µl of eluate to column, 1min incubation, Spin 8,000rpm for 1min
22) Discard columns and apply labels
23) Nanodrop (blank with AE buffer)
24) store at -20°C until PCR analysis (Immuno Freezer 4, 5th drawer)

Sample code	Sample type	Cell count	Elution volume	Ng/µl	260/280 ratio	260/230 ratio	Comment
1P	PBMC						
2P	PBMC						
3P	PBMC						
4P	PBMC						
5P	PBMC						
6P	PBMC						
7P	PBMC						
8P	PBMC						
9P	PBMC						
10P	PBMC						
11P	PBMC						
12P	PBMC						
1R	Naïve CD4						
2R	Naïve CD4						
3R	Naïve CD4						
4R	Naïve CD4						
5R	Naïve CD4						
6R	Naïve CD4						
7R	Naïve CD4						
8R	Naïve CD4						
9R	Naïve CD4						
10R	Naïve CD4						
11R	Naïve CD4						
12R	Naïve CD4						

APPENDIX XI: Standard operating procedure for TREC and KREC Quantification

Reagents and consumables:

- PCR grade Water – Sigma, Part No. W4502 1 Litre Bottle.
- 100 x TRIS-EDTA (TE) buffer – Sigma, Part No. T-9285 100ML bottle.
- Patient Naïve T- cells (stored at -20°C)
- Standards (stored at -20°C)
- FAST optical 96-well reaction plate
- Micro Amp adhesive film
- Azowipes – VWR International. Part No. 222/01 55/02.
- 1.5mL autoclaved snap top micro centrifuge tubes.
- Pipette tips (autoclaved).

MASTER-MIX (measures given for each well required):

All primers and probes should be stored at -20°C in the dark, diluted as indicated by the manufacturer then separated into aliquots:

- TREC F Primer (45 µM) - 0.5µl
- TREC R Primer (45 µM) – 0.5µl
- TREC FAM-TAMRA Probe (10µM) – 0.5µl
- KREC F Primer (45 µM) – 0.5µl
- KREC R Primer (45 µM) – 0.5µl
- KREC HEX-TAMRA Probe (10µM) – 0.5µl
- TRAC F Primer (45 µM) – 0.5µl
- TRAC R Primer (45 µM) – 0.5µl
- TRAC FAM-TAMRA Probe (10µM) – 0.5µl
- Sterile water – 4.5 or 6µl (kept at room temperature)
- Taqman FAST UMM polymerase (2x) : 12.5µl (stored at 4°C)

For DNA extraction using the mini-kit:

- Buffers AE, AW1, AW2, AL
- QIAGEN Protease
- Ethanol 96-100%
- PBS
- 2ml collection tubes
- QIAamp Mini spin columns

Equipment: Taqman PCR machine PTC 100, program 7500

Sample requirement and storage: Cell pellets from separated T cell fractions and stored at -20°C. Cell pellets are stable for DNA extraction for 12 mths at -80°C.

Methods

A: DNA Extraction (Appendix X: Thymic Output SOP)

B: Preparing the Taqman plate

C: Running the Taqman plate

D: Notes on analysis

E: Making the standard curve

B: Preparing the Taqman plate

1) Defrost samples from -20°C in room temperature. Each sample requires 35µl of extracted DNA. Remove standards out of freezer.
2) Label Taqman plate/chart to reflect the 96 well plate:
    a. 3 of each of the 5 standards for TRECs/KRECs and 2 x ($1x10^6$), 3 x 5 for for TRACs standards and 2 x ($1x10^6$) – therefore 34 wells for standards
    b. 6 of each sample – maximum 10 samples per plate (60 wells)
    c. 2 blanks
3) Calculate quantities of probes/primers needed for the mastermixes. Always calculate total requirements for two extra wells.
4) Prepare the Mastermixes in PCR clean room. Multiply by number of wells. Ensure enough is made for each of the wells – a total of 20µl of the Mastermix and 5µl of standard solution or 5µl of patient DNA will go into each well (excluding the blank wells which will have 5µl sterile water). Therefore each well will contain 20µl of solution.

MASTER-MIX for TRECs/KRECs:

Reagent	Conc	Volume/well	Volume for x+2 extra wells
TREC F Primer	45µM	0.5µl	
TREC R Primer	45µM	0.5µl	
TREC FAM-TAMRA Probe	10µM	0.5µl	
KREC F Primer	45µM	0.5µl	
KREC R Primer	45µM	0.5µl	
KREC HEX-TAMRA Probe	10µM	0.5µl	
Water	-	4.5µl	
Taqman UMM	2x	12.5µl	

MASTER-MIX for TRACs:

Reagent	Conc	Volume/well	Volume for x+2 extra wells
TRAC F Primer	45µM	0.5µl	
TRAC R Primer	45µM	0.5µl	
TRAC FAM-TAMRA Probe	10µM	0.5µl	
Water	-	6µl	
Taqman UMM	2x	12.5µl	

5) Vortex Mastermixes for 15 seconds
6) Apply 20µl of TRECs/KRECs Mastermix to the 1st 48 wells (base of each well).
7) Apply 20µl of TRACs Mastermix to the next 48 wells (bottom half)
8) Take to DNA prep room with samples and standards
9) Remove standards from fridge – 5 standards. The 6th will need to be made fresh each time.

Tube 1	10^6 copies/5µl
Tube 2	10^5 copies/5µl
Tube 3	10^4 copies/5µl
Tube 4	10^3 copies/5µl
Tube 5	100 copies/5µl
Tube 6	Empty

10) Prepare Tube 6: take 3µl of tube 5 and add to 27µl sterile water = 10 copies/5µl
11) Vortex Standards or pipette mix
12) Pipette 5µl of standard solution into each of the standard wells – see worksheet (i.e. $2x10^6$, $3x10^5$, $3x10^4$, $3x10^3$, $3x10^2$, 3x10 = 17 wells for top half of tray TRECs/KRECs… and 17 wells for bottom half: TRACs).
13) Pipette 5µl of sterile water to the two blank wells.
14) Add 5µl of each extracted DNA sample to the specimen wells – 3 per TRECs/KRECs half of the plate and 3 each per the TRACs half. Therefore max 10 samples.
15) Attach the microAmp adhesive film to the plate and score with a Micro Amp adhesive film applicator
16) Cover with foil and keep in fridge if any delays.
17) Check wells for air-bubbles. If any present, VERY gently tap underneath.

NB. Ideally also have a “run control” with each run.

C: Running the Taqman plate

1) Spin the plate at 3500rpm for 1min (or 4680rpm if any air bubbles).
2) Set up computer
    a. 7500 software, login as guest-user
    b. Place plate in PCR machine
    c. Select template kept under file name “Helen”
    d. Plate set-up: TRECs/KRECs and TRACs standards already set, as are blanks. Add number of patient samples (max. 10)
    e. Number each well in triplets in both halves of the plate (tick the patient sample box then highlight the wells)

f. Highlight all patient wells and tick the box for TRECs/KRECs or TRACs – depending on side of plate.
g. Select “S” for standards, “N” for blanks and “U” for samples
h. Set cycles to 45-50 (it measures from 22-50 cycles)
i. Check threshold of 0.03
j. Save plate under date and code for samples
k. Start Run… –will take approximately 2hrs
l. At end of run click OK at the prompt and save

D: Notes on analysis

- Set threshold at 0.03 or closest per each TREC, KREC or TRAC analysis that matches the ideal ct mean
- Set cycles at 3-points lower (ie. If 20 cycles, set at 17)
- Save raw data in separate file
- Review the standard curves for slope, intercept and R2 values (eyeball which points, if any, may need to be omitted)

Slope	Range must be within -3.1-3.6 (ideally 3.3)
Intercept	Should be consistent but range could be 36-41
R2	>0.97
Ct value	Should typically fall within +2 points of the average range

- Check each triplet of standards and complete the table (amplifications, omissions, ct mean etc). Check samples triplets (if any 1 deviates – re-run sample)
- Omit wells and reanalyse (only up to 3 data points can be removed from stds)
- Check standard curve meets requirements
- Then, record results from samples
- Save analysed data and separate file - export to excel
- Blanks should have no peaks
- All standards should have peaks at spaced intervals
- Each sample should have a TRAC peak but may/may not have a TREC/KREC peak depending on the clinical condition
- Expected Reference Range:

Newborn-3yrs	CD3+ T cells >100,000
3yrs-10yrs	CD3+ T cells 30,000-70,000
10yrs-16yrs	CD3+ T cells 20,000-60,000
Adult	Negligible TREC levels

- Expected Reference Range: 10-1000ng/µl DNA concentration
- Expect 0.044 TRECs/cell (from TRECs/No. cells from TRAC)

E: Making the standard curve

	Stock = 2 x 10^6/µl (10^7/5µl)	This should be enough for 60 runs
Standard 1	1 x 10^6/5µl	150µl stock + 1350µl buffer
Standard 2	1 x 10^5/5µl	200µl of 1 + 1800µl buffer
Standard 3	1 x 10^4/5µl	200µl of 2 + 1800µl buffer
Standard 4	1 x 10^3/5µl	200µl of 3 + 1800µl buffer
Standard 5	1 x 10^2/5µl	200µl of 4 + 1800µl buffer
Standard 6	10/µl	Make fresh every time

Quantity	Mean Ct TRECs	Mean Ct KRECs	Mean Ct TRACs
10	35.46	35.17	35.13
100	32.15	31.79	31.90
1,000	28.83	28.83	28.50
10,000	25.43	25.10	25.23
100,000	22.02	21.70	21.82
1,000,000	18.21	18.25	18.20

APPENDIX XII: Standard operating procedure for quantitative anti-gp120 antibody ELISA

Concept:

Two ELISAs plates run together – one will measure gp120-specific IgG and the other will measure total IgG. Therefore the output may be described as a ratio of gp120-specific IgG per total IgG. It will also be possible to estimate the child's total IgG.

Reagents Required:

- Anti-Human IgG (Fc Specific) Antibody Produced in Goat, Affinity Isolated Antibody, Buffered Aqueous Solution (for coating the plate)
- Goat anti-Human, IgA+G+M (H+L), Biotin (detection antibody)
- Anti-Biotin-Peroxidase Antibody Produced in Goat, Affinity Isolated Antibody, Buffered Aqueous Solution (capture antibody)
- IgG from Human Serum, Reagent Grade, >95% (for the IgG standard curve)
- Goat Serum, Donor Herd, USA Origin, Sterile- Filtered, Suitable for Cell Culture (for blocking agent/solution for IgG ELISA)
- Tween® 20, viscous liquid (for various buffer solutions)
- Plate Immuno F96 MaxiSorp (Cert) Polystyrene
- Carbonate-bicarbonate (for plate) Sigma: C3041-50 CAP
- Plate Sealer, EASYsealTM, Transparent
- Sulphuric acid (1.0 molar) – for stopping agent
- Non-fat dry milk (Elite) – for buffers

Preparations of buffers / agents:

- Carb-bicarbonate: Break one tablet into 100ml distilled water. Keep at 4°C for one month.
- PBS/Tw20: 1 litre PBS with 500µl Tween20 (for 2 plates). Make fresh each time.
- Blocking agent for IgG plate: 5% goat serum, 5% milk, 0.05% Tween20 and PBS. Make fresh each time. Make 30mls per IgG plate e.g. 30ml PBS, 1.5ml goat serum, 1.5g milk powder, and 15µl Tween20.
- Dilution buffer: PBS, 5% milk, 0.05% Tween20. Make fresh each time. Make 50mls per gp120 plate e.g. 50ml PBS, 2.5g milk powder, 25µl Tween20.

Preparation of the plates:

IgG Plate	Gp120 plate
1) Make IgG coating buffer: add anti-human IgG to carb-bicarbonate to make a solution of 4ug/ml. 11µl of anti-h IgG and 6ml carb-bicarbonate.	1) Prepare gp120 coating buffer: 8µl of 5mg/ml gp120 in 10mls of Carb-Bicarbonate buffer.
2) Coat plate with anti-human IgG in coating buffer, 50µl per well.	2) Coat plate with gp120 coating buffer, 50µl per well.
3) Incubate overnight at 4°C or for one hour at 37°C.	
4) Wash x 4 in PBS/Tw20 solution.	
5) Block with 200µl blocking agent.	5) Block with 100µl dilution buffer.
6) Incubate 1hr at 37°C.	
7) Calculate **sample dilution series and prepare standard curve** and samples in separate sample plate. NB, for IgG plate the samples and standards must be mixed in the goat-serum blocking agent and for the gp120 plate the standards and samples must be mixed with the dilution buffer. See dilution series template example below.	
8) Wash x 4 in PBS/Tw20 solution.	
9) Add human IgG standard to top of scale and proceed to do serial dilution. 3µl in 300µl goats serum mix.	9) Add gp120 to top of scale and proceed to do serial dilution. 9µl in 900µl dilution buffer.
10) Add samples to IgG plate diluted in goats serum blocking agent. Proceed to do serial dilution according to scale of dilution series e.g. 1:50 (3µl in 150µl).	10) Add samples to gp120 plate diluted in dilution buffer. Proceed to do serial dilution according to scale of dilution series.
11) Add 50ul of the sample dilution from the sample plate to each the IgG and gp120 ELISA plates	
12) Incubate 1hr at 37°C.	
13) Wash x 4 in PBS/Tw20 solution.	
14) Add goat anti-human total Immunoglobulin-Biotin titrated to 1:1000. 4µl antibody in 12mls dilution buffer, 50µl per well	
15) Incubate 1hr at 37°C.	
16) Wash x 4 in PBS/Tw20 solution.	
17) Add Anti-biotin-peroxidase antibody produced in goat 50µl per well (optimal concentration 1:1000)	
18) Incubate 1hr at 37°C, remove TMB from fridge.	
19) Wash x 4 in PBS/Tw20 solution.	

20) Add 100µl TMB substrate and wait 2-5 mins for colour to develop.
21) Stop with 25µl of H2SO4 (1 molar).
22) Read on ELISA plate reader at 450nm (NB. Plates stable to be re-run for 15mins).

Dilution series:

For standard curve – use from 4 to 16 standards. Fill top well only with standard and dilution e.g. 3µl human IgG and 300µl goats serum blocking agent.
For a 3-fold dilution series:
- Fill the remaining wells with 60µl dilution buffer
- Take 30µl from top well (Std1) and add to second well (Std2).
- Mix 10x to resuspend the solution.
- Take 30µl from the second well and add to the third (Std3) etc.
- No need to change tips.
- Example: for a 4-fold solution, fill each well with 90µl and add 30µl to mix.

For the samples – use 8 dilutions or more. Use judgment to estimate the scale of dilution (initial and fold-series). Note IgG will always be far more concentrated than gp120 and therefore will need more dilution i.e. 5 or 6-fold dilution whereas gp120 may only require 3-fold dilution. As per standards, fill top well only with standard and dilution e.g. 3µl sample in 150µl dilution buffer.

Std1	Std9	Sample1	Sample2	3	4	5	6	7	8	9	10
Std2	Std10	Sample1	Sample2								
Std3	Std11	Sample1	Sample2								
Std4	Std12	Sample1	Sample2								
Std5	Std13	Sample1	Sample2								
Std6	Std14	Sample1	Sample2								
Std7	Std15	Sample1	Sample2								
Std8	Std16	Sample1	Sample2								

Dilution series by plate:

IgG Plate:

2/198	30/60	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000	1/1000
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	1/1000
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	1/1000
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	1/1000
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	

gp120:

5/95	30/60	3/147	3/147	3/147	3/147	3/147	3/147	3/147	3/147	3/147	3/147
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	3/147
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	3/147
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	3/147
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	
30/60	30/60	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	30/120	

Setting up the ELISA reader and interpreting the results:

- Computer will calculate the dilution series, only the labels and scale of dilution need to be entered.
- Standard curve must be visually sigmoid, one or two points may be excluded if necessary. No official parameters to be met.

Storage and Aliquots:

Item	Aliquot size (Enough for 1 plate)	Storage	Function
Anti-Human IgG	12µl	-20°C	Plate-coating
Goat anti-human total Ig Biotin	4.5µl	-20°C	Detection antibody
Anti-biotin peroxidase	12µl	-20°C	Capture antibody
IgG from Human Serum	3.5µl	-20°C	IgG standard curve
Goat serum	2ml	-20°C	For buffers
GP120 clade C monoclonal antibody	9.5µl	-20°C	For standard curve

APPENDIX XIII: Standard operating procedure for HIV proviral DNA quantification

Reagents/Equipment:

PDHTaq1 primer	5' -TGA AAG TTA TAC AAA ATT GAG GTC ACT GTT -3'
PDHTaq2 primer	5'- TCC ACA GCC CTC GAC TAA CC -3'
PDHTaqPR probe	5'- JOE-CCC CCA GAT ACA CTT AAG GGA TCA ACT CTT AAT TGT TAMRA-3'
HIV1LTRTaq1 primer	5'- GCC TCA ATA AAG CTT GCC TTG A- 3'
HIV1LTRTaq2 primer	5' -GGC GCC ACT GCT AGA GAT TTT- 3'
HIV1TaqPR probe	5'- FAM -TGT GAC TCT GGT AAC TAG AGA TCC CTC AGA C TAMRA-3'

- Qiagen EZ-1 robot and virus kit
- Applied Biosystems Taqman 7500
- Abgene PCR plates Thermo-fast 96 non-skirted clear (Cat no: AB-0600^)
- Abgene Absolute QPCR plate seal (Cat no: AB-1170)
- Gilson Pipettes and barrier tips (p2/p20/p200/p1000)
- Qiagen carrier RNA
- Qiagen Multiplex PCR Master Mix (with ROX dye) Kit (Cat no: 204543)
- Mol. Grade nuclease free water
- Extracted DNA from 8E5 cell line
- Use between 10,000 and $5x10^6$ cells (use carrier DNA if <10,000 cells)
- 100,000 cells yields 600ng DNA (PCR max)

After DNA extraction record nanodrop results in the following table and calculate precise volume of DNA extract to achieve maximum sensitivity of the assay by quantifying 600ng of DNA per reaction well.

Sample ID	Nanodrop (ng/ul)	260/280	260/230	Vol for 600ng = 600ng/sample	Vol H20 to make 11µl

- A non-template control for every run
- Each standard and sample to be run in triplicate with average values used
- Samples were re-run when less than the 3 reactions amplified or values differed by greater than 2 sd.
- The explicit quantity of provirus per 10^5 PBMCs is the output value of LTR divided by PDH.

Quantitative HIV Proviral PCR

- Carrier RNA, Standards, Samples, Qiagen mix, Mol H20, Plate and adhesive, Primers/probes

8E5 standard curve (e.g. 8E5 stock = 1x10^5/µl)
Make the 1:10 dilution series using Carrier RNA: 5ug/ml so do a 1 in 200 dilution = 1.5µl + 298.5µl nuclease-free water. For example:

1. Std 1 = 10^5 = 3.4µl stock + 30.6µl carrier RNA
2. Std 2 = 10^4 = 4µl Std 1 + 36µl carrier RNA
3. Std 3 = 10^3 = 4µl Std 2 + 36µl carrier RNA
4. Std 4 = 10^2 = 4µl Std 3 + 36µl carrier RNA
5. Std 5 = 10 = 4µl Std 4 + 36µl carrier RNA
6. Std 6 = 1 = 4µl Std 5 + 36µl carrier RNA

Make the 25µl PCR mastermix

a. **Make working stock**
b. **Make mastermix**
c. **Add mastermix to plate (14µl per well)**
d. **Add water to standards, NTC and one sample well**
e. **Add standards**
f. **Add sample DNA**

Reagent	**Volume per well**	**Working stock required**	**Stock [100µM] and calculation to get to working [10µM]**	**Final concentration**	**Volume x __wells**
PDH forward primer	0.25µl	10µM	**…µl working stock + …µl H20**	100nM	
PDH reverse primer	0.25µl	10µM	**…µl working stock + …µl H20**	100nM	
PDH probe	0.25µl	10µM	**…µl working stock + …µl H20**	100nM	
HIV1LTRTaq 1 primer	0.25µl	**20µM**	**…µl working stock + …µl H20**	**200nM**	
HIV1LTRTaq 2 primer	0.25µl	**20µM**	**…µl working stock + …µl H20**	**200nM**	
HIV1TaqPR probe	0.25µl	10µM	**…µl working stock + …µl H20**	100nM	
Qiagen MM	12.5µl	2 x	-	1x	
8E5 Stds	11µl total (10µl 8E5 +1µl H_20)				
600ng DNA	11µl total (DNA+H20)				

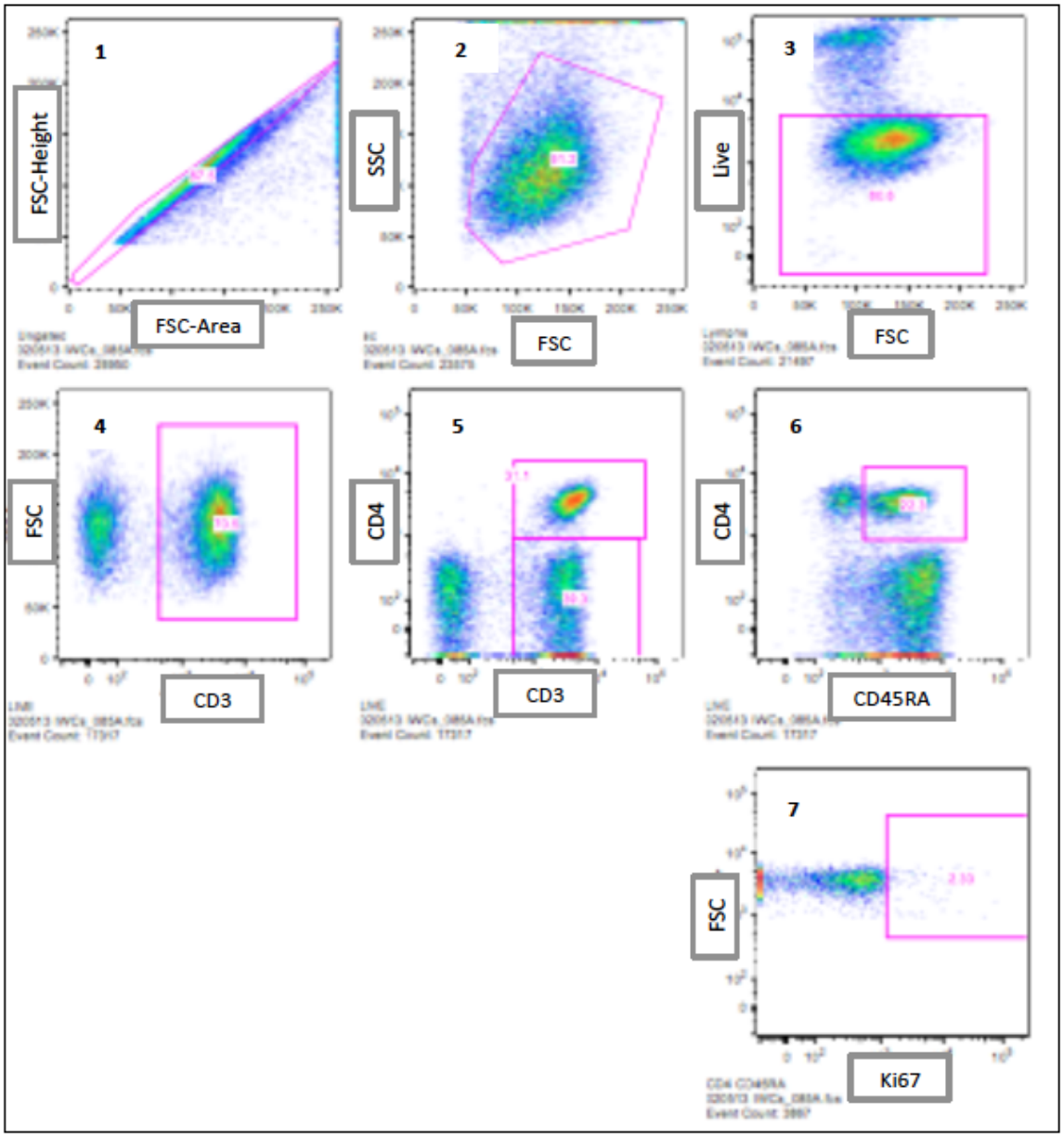
Set up PCR **Taqman** / Applied Biosystems7500 :

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Dyes: Joe and Tamra (PDH)
Fam and Tamra (LTR)

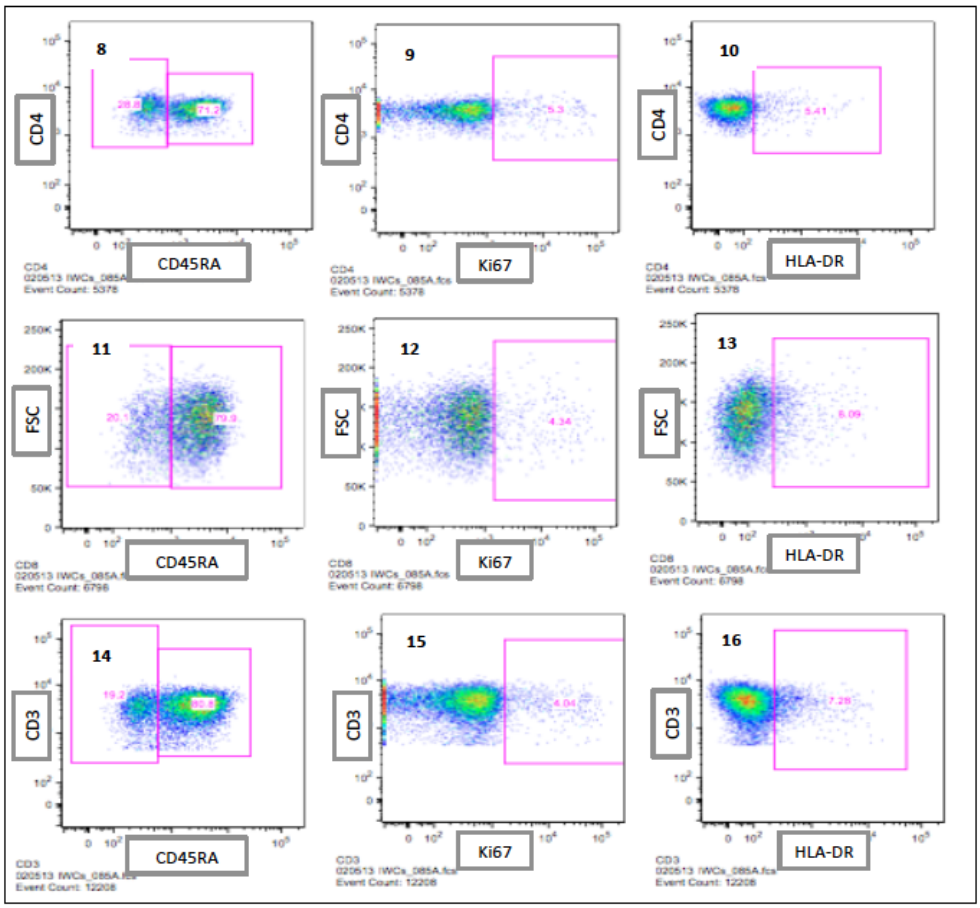
Cycling: 95°C for 15 mins x 45
95°C for 60s
60°C for 60s

APPENDIX XIV: Gating strategy for Panel A



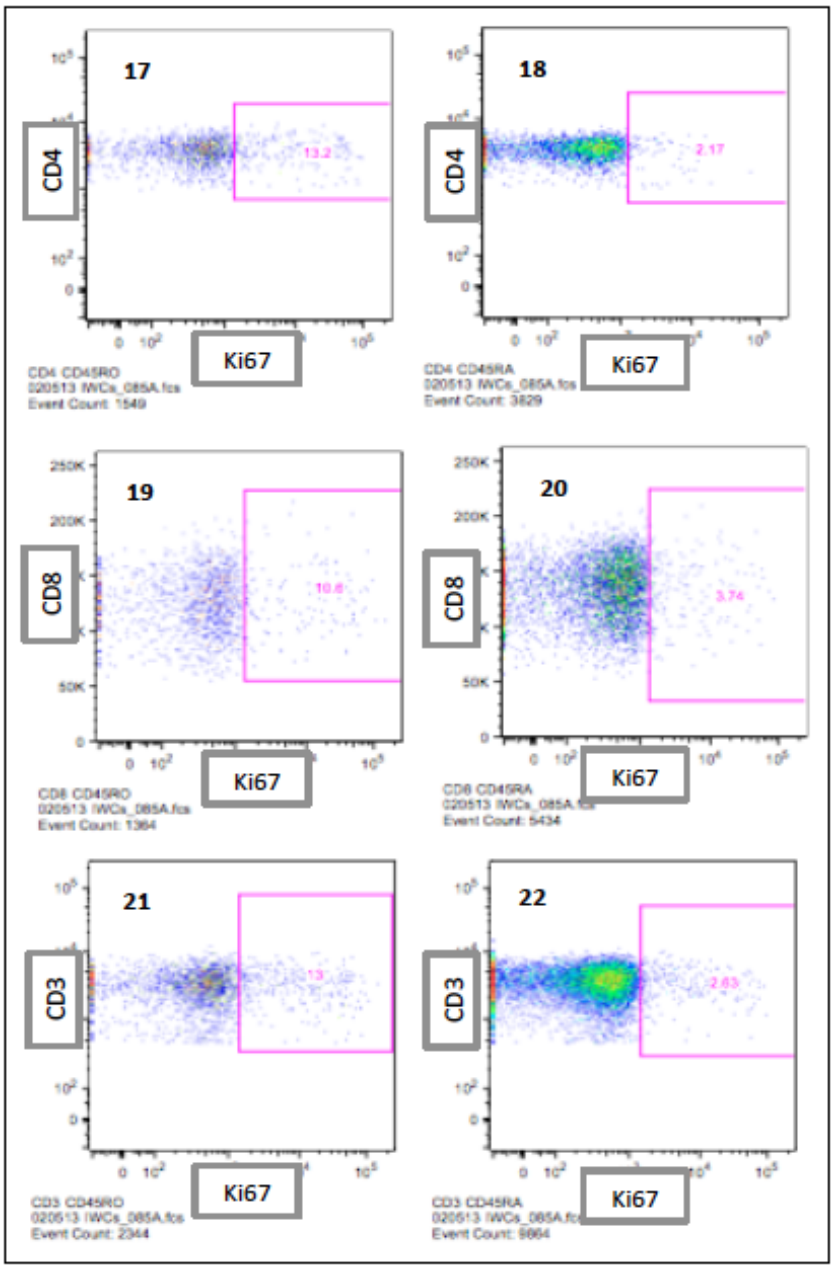
From left to right (plots 1-7):

1) Forward-height versus forward-area scatter, gated on single cells.
2) From single cells on plot 1: Forward versus side scatter, gated on lymphocytes.
3) From lymphocytes on plot 2: Forward scatter on x-axis, viabliity marker on y-axis, gated on live cells (%viability).
4) From live cells on plot 3: Forward scatter versus CD3-PECy7, gated on CD3+ (CD3%).
5) From plot 3: CD4-PerCP on x-axis versus CD3-PECy7 on y-axis, gated on CD4+CD3+ (CD4%) and CD4-CD3+ (CD8%).
6) From plot 3: CD4-PercP on x-axis versus CD45RA-FITC on y-axis, gated on CD4+CD45RA+ lymphocytes (naïve CD4+ T-cells).
7) From plot 6: Ki67-PE on x-axis versus forward scatter, gated on CD4+CD45RA+Ki67+ lymphocytes (%proliferation to be used in thymic output formula).



From left to right (plots 8-16):

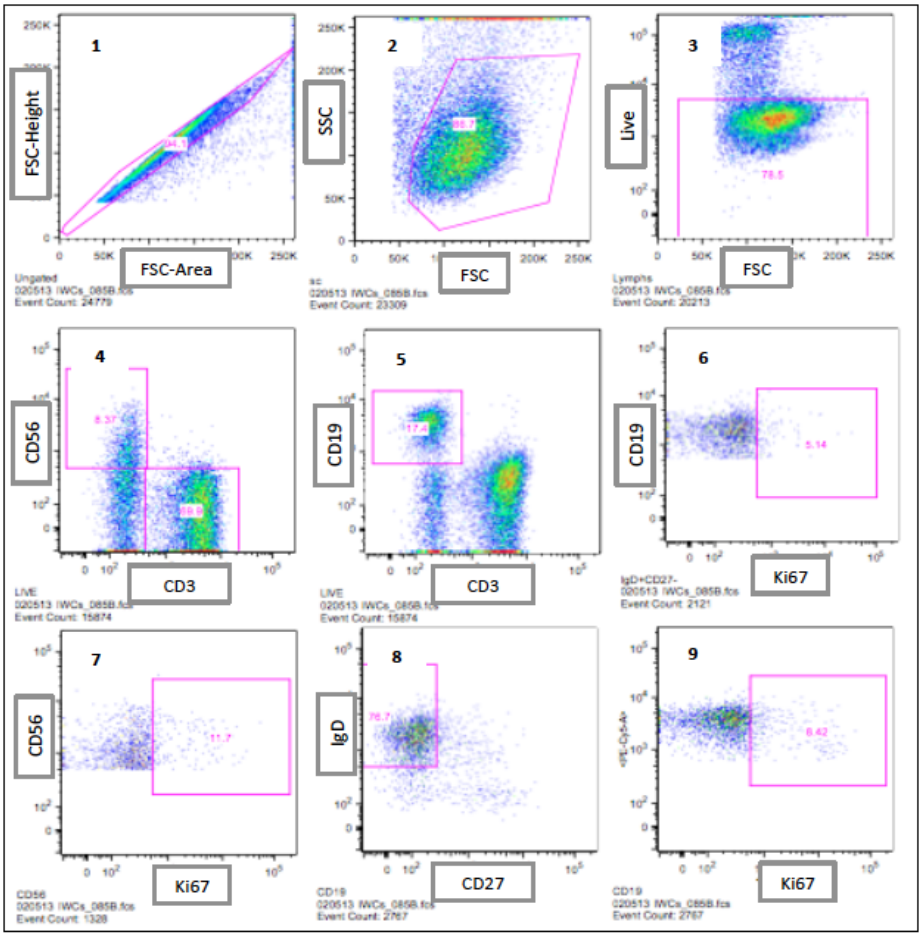
8) From CD4 population on plot 5: CD45RA-FITC on x-axis versus CD4-PerCP on y-axis, gating on CD4+CD45RA- (left, CD4 memory) and CD4+CD45RA+ (right, CD4 naive).
9) From CD4 population on plot 5: Ki67-PE on x-axis versus CD4-PerCP on y-axis, gating on CD4+ Ki67+ lymphocytes.
10) From CD4 population on plot 5: HLADR- pacific blue on x-axis versus CD4-PerCP on y-axis, gating on CD4+HLADR+ lymphocytes.
11) From CD8 population on plot 5: CD45RA-FITC on x-axis versus forward scatter on y-axis, gating on CD3+CD4-CD45RA- (left, equivalent CD8 memory) and CD4+CD45RA+ (right, equivalent CD8 naive).
12) From CD8 population on plot 5: Ki67-PE on x-axis versus forward scatter on y-axis, gating on CD3+CD4-Ki67+ lymphocytes (equivalent CD8 Ki67).
13) From CD8 population on plot 5: HLADR-pacific blue on x-axis versus forward scatter on y-axis, gating on CD3+CD4-HLADR+ lymphocytes (equivalent CD8 HLADR).
14) From CD3 population on plot 4: CD45RA-FITC on x-axis versus CD3-PECy7 on y-axis, gating on CD3+CD45RA- (left, CD3 memory) and CD3+CD45RA+ (right, CD3 naive).
15) From CD3 population on plot 4: Ki67-PE on x-axis versus CD3-PECy7 on y-axis, gating on CD3+Ki67+ lymphocytes.
16) From CD3 population on plot 4: HLADR- pacific blue on x-axis versus CD3-PECy7 on y-axis, gating on CD3+HLADR+ lymphocytes.



From left to right (plots 17-22):

17) From CD4+CD45RA- population on plot 8: Ki67-PE on x-axis versus CD4-PerCP on y-axis, gated on CD4+CD45RA-Ki67+ lymphocytes (proliferation of memory CD4).
18) From CD4+CD45RA+ population on plot 8: Ki67-PE on x-axis versus CD4-PerCP on y-axis, gated on CD4+CD45RA+Ki67+ lymphocytes (proliferation of naïve CD4).
19) From CD4-CD45RA- population on plot 11: Ki67-PE on x-axis versus forward scatter on y-axis, gated on CD3+CD4-CD45RA-Ki67+ lymphocytes (proliferation of memory CD8).
20) From CD4-CD45RA+ population on plot 11: Ki67-PE on x-axis versus forward scatter on y-axis, gated on CD3+CD4-CD45RA+Ki67+ lymphocytes (proliferation of naive CD8).
21) From CD3+CD45RA- population on plot 14: Ki67-PE on x-axis versus CD3-PECy7 on y-axis, gated on CD3+CD45RA-Ki67+ lymphocytes (proliferation of memory CD3).
22) From CD3+CD45RA+ population on plot 14: Ki67-PE on x-axis versus CD3-PECy7 on y-axis, gated on CD3+CD45RA+Ki67+ lymphocytes (proliferation of naive CD3).

APPENDIX XV: Gating strategy for Panel B



From left to right (plots 1-9):

1) Forward-height versus forward-area scatter, gated on single cells.
2) From single cells on plot 1: Forward versus side scatter, gated on lymphocytes.
3) From lymphocytes on plot 2: Forward scatter on x-axis, viability marker on y-axis, gated on live cells (%viability).
4) From live cells on plot 3: CD3-FITC on x-axis versus CD56-PECy7 on y-axis, gated on CD3-CD56+ (NK-cells) and CD3+CD56- (T-cells).
5) From live cells on plot 3: CD3-FITC on x-axis versus CD19-PerCP on y-axis, gated on CD3-CD19+ (B-cells).
6) From B-cells on plot 5: Ki67-PE on x-axis versus CD19-PerCP on y-axis, gated on CD19+Ki67+ lymphocytes (proliferation of B-cells).
7) From NK-cells on plot 4: Ki67-PE on x-axis versus CD56-PECy7 on y-axis, gated on CD56+Ki67+ lymphocytes (proliferation of NK-cells).
8) From B-cells on plot 5: CD27-BV421 on x-axis versus IgD-BV510 on y-axis, gated on CD19+IgD+CD27- lymphocytes (naïve, unswitched B-cells).
9) From naïve unswitched B-cells on plot 8: Ki67-PE on x-axis versus IgD-BV510 on y-axis, gated on CD19+IgD+CD27-Ki67+ (proliferation of naïve, unswitched B-cells).

APPENDIX XVI: Publication 1

Observed full blood count and lymphocyte subset values in a cohort of clinically healthy South African children from a semi-informal settlement in Cape Town.

South African Medical Journal
July 2015; 105(7): 589-595